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# Decreased IFNγ Message by Pancreatic Islet-Infiltrating Leukocytes is a Common Feature of Different Treatments that Protect Against Autoimmune Diabetes in the BB Rat

by

AMR ABDELHAMID FARAG EL-SHEIKH ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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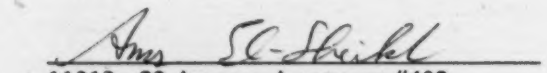

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# ABSTRACT

Autoimmune diabetes results from a selective destruction of the insulin-producing pancreatic islet-β-cells. The disease is thought to result from a complex interaction between the environment of the individual and his/her genetic predisposition. The genetic composition of the diabetes-susceptible individual is believed to affect the development and/or responsivity of the immune system, possibly to environmental factors, resulting in an autoimmune response causing the selective destruction of the insulin-producing β-cells.

T-cells and their products (cytokines) are considered the central mediators of immune responses. The current classification of cytokines (e.g. the mutually inhibitory type 1 and type 2) is based on the response they produce. The development of IDDM is thought to involve a bias toward type 1 cytokines in the predisposed individual. This thesis is mainly concerned with investigating expression of cytokines during autoimmune diabetes using the BB rat as a model and seeking associations and correlations between cytokine patterns (type 1 or 2), as well as cytotoxic molecules, and β-cell destruction.

Steady-state mRNA levels were investigated in the islets (and spleens) of BB rats during spontaneous diabetes and during the protection provided by immunostimulatory (CFA) and

pharmacotherapeutic (theophylline) interventions. Also, the steady-state mRNA levels of cytokines, and cytotoxic molecules, were investigated in the diabetes-prone BB rat after selective deletion of $CD4^+$ and $CD8^+$ T-cells by administration of anti-T-cell mAbs. My studies (CFA and mAb studies) show strong association between the steady-state levels of type 1 cytokine messages, most importantly IFNγ, and the development of diabetes. Furthermore, strong correlation was found between the steady-state mRNA levels of type 1 cytokine, IFNγ, and that of perforin during the development of diabetes, and during the protection conferred by administration of mAbs directed against $CD4^+$ and $CD8^+$ T-cells. On the other hand, there was no consistent association found between the steady-state mRNA levels of type 2 cytokines (IL-4 and IL-10) and the protection against diabetes. There was also no correlation between the steady-state mRNA levels of type 2 cytokines.

**To my parents**

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LIST OF ABBREVIATIONS

| | |
|---|---|
| aa: | amino acid |
| Ab: | antibody |
| ACTH: | adrenocorticotropic hormone |
| AD: | acutely diabetic |
| ADCC: | antibody-dependent cell cytotoxicity |
| allo-: | allogeneic |
| APC: | antigen presenting cells |
| Asp: | aspartate |
| BB: | Biobreeding |
| BCG: | *Bacillus* Calmette-Gurin |
| BM: | bone marrow |
| BN: | Brown Norway |
| bp: | bone pain |
| BSA: | bovine serum albumin |
| Ca: | calcium |
| cAMP: | cyclic adenosine monophosphate |
| CD: | cluster designation |
| cDNA: | complementary DNA |
| CFA: | complete Freund's adjuvant |
| cm: | centimetre |
| $CO_2$: | carbon dioxide |
| Con A: | concanavalin A |
| CsA: | cyclosporine A |
| CSF-1: | colony stimulating factor 1 |
| CTL: | cytotoxic T lymphocyte |
| Cy: | cyclophosphamide |
| Cyp: | cyclophilin |
| DC: | dendritic cells |
| DNA: | deoxyribonucleic acid |
| DM: | diabetes mellitus |
| DOC: | deoxycorticosterone |
| DP: | diabetes-prone |
| DR: | diabetes-resistant |
| DTH: | delayed-type hypersensitivity |
| EAE: | experimental allergic encephalomyelitis |
| EC: | endothelial cells |
| EDTA: | ethanolamine diamine tetra-acetic acid |
| ELISA: | enzyme-liked immunosorbent assay |
| FBS: | fetal bovine serum |
| FITC: | fluorescein isothiocyanate |
| g: | grams |

LIST OF ABBREVIATIONS CONT'D:

| | |
|---|---|
| GAD: | glutamic acid decarboxylase |
| G-CSF: | granulocyte colony stimulating factor |
| GKO: | gene knockout |
| GM-CSF: | granulocyte-macrophage colony stimulating factor |
| HBS: | Hank's balanced saline solution |
| HA: | human leukocyte antigen |
| Hcl: | hydrochloric acid |
| HPA: | hypothalamic-pituitary-adrenal axis |
| hr: | hour |
| HSLAS: | Health Sciences Laboratory Animal Services |
| hsp: | heat shock protein |
| Ia: | Class II major histocompatibility complex antigen |
| ICA: | islet-cell antibody |
| ICAM-1: | intercellular adhesion molecule 1 |
| ICSA: | islet cell surface antibody |
| IDDM: | insulin-dependent diabetes mellitus |
| IFA: | incomplete Freund's adjuvant |
| IFN-: | interferon- |
| Ig: | immunoglobulin |
| IgG: | immunoglobulin G |
| IgM: | immunoglobulin M |
| I-HPA: | immune-hypothalamic-pituitary-adrenal axis |
| IL: | interleukin |
| IMNL: | islet mononuclear leukocyte |
| iNOS: | inducible nitric oxide synthase |
| i.p.: | intraperitoneal |
| IRF: | interferon regulatory factor |
| JAK: | Janus kinase |
| kDa: | kilodaltons |
| kg: | kilogram |
| L: | litre |
| LCA: | leukocyte common antigen |
| LCMV: | lymphocytic choriomeningitis virus |
| LPS: | lipopolysaccharide |
| LT: | lymphotoxin |
| Lyp: | lymphopenia locus |
| M: | molar |
| mAb: | monoclonal antibody |
| MAC: | membrane attack complex |
| MGP: | myelin basic protein |
| M-CSF: | macrophage colony stimulating factor |

LIST OF ABBREVIATIONS CONT'D:

| | |
|---|---|
| MHC: | major histocompatibility complex |
| mg: | milligram |
| Mg: | magnesium |
| mL: | millilitre |
| MLC: | mixed lymphocyte culture |
| MLR: | mixed lymphocyte reaction |
| mmol: | milli-molar |
| MNL: | mononuclear leukocyte |
| mRNA: | messenger ribonucleic acid |
| NFAT: | nuclear factor activating transcription |
| NF-κB: | nuclear factor-kappa B |
| ng: | nanogram |
| NK: | natural killer |
| NKCF: | natural killer cytotoxic factor |
| NKSF: | natural killer stimulatory factor |
| nm: | nanometer |
| NO: | nitric oxide |
| NOD: | non-obese diabetic |
| NOS: | nitric oxide synthase |
| O.D.: | optical density |
| p: | protein |
| *p*: | probability |
| PBS: | phosphate-buffered saline |
| PCR: | polymerase chain reaction |
| PD: | prediabetic |
| PDE: | phosphodiesterase |
| pg: | picogram |
| PGE: | prostaglandin E |
| PHA: | phytohemaglutinin |
| PKC: | protein kinase C |
| PLA: | phospholipase A |
| PLC: | phospholipase C |
| PMA: | phorbol myristate acetate |
| poly I:C: | poly inosinic-polycyctidylic acid |
| psl: | photo-stimulated luminescence |
| PTX: | pentoxyphylline |
| r: | regression coefficient |
| RFLP: | restriction fragment length polymorphism |
| rh: | recombinant human |
| RIA: | radio-immunoassay |
| rpm: | revolutions per minute |

LIST OF ABBREVIATIONS CONT'D:

| | |
|---|---|
| RT: | reverse transcription |
| RT1: | rat major histocompatibility complex |
| SCID: | severe combined immune deficiency |
| S.E.: | standard error of the mean |
| sIg: | surface immunoglobulin |
| SMLR: | syngeneic mixed lymphocyte reaction |
| SRBC: | sheep red blood cells |
| SV40: | sarcoma virus 40 |
| STAT: | signal transduction and activators |
| TCR: | T lymphocyte receptor |
| $T_{c/s}$: | T cytotoxic/suppressor |
| TGF: | transforming growth factor |
| $T_h$: | T helper |
| Theo: | theophylline |
| TNF: | tumour necrosis factor |
| TNF-R: | tumour necrosis factor receptor |
| TYK: | tyrosine kinase |
| U: | unit |
| µg: | microgram |
| µL: | microlitre |
| µm: | micrometer |
| µM: | micromolar |
| unstim: | unstimulated |
| Vβ: | beta chain variable region of T cell receptor |
| VAF: | viral antigen-free |
| VCAM-1: | vascular cell adhesion molecule 1 |
| v/v: | volume/volume |
| vs.: | versus |
| WF: | Wistar Furth |
| w/v: | weight/volume |
| Wor: | Worcester |

# Chapter 1

# Introduction

# I. Insulin-Dependent Diabetes Mellitus

Diabetes Mellitus (DM) is a syndrome characterized by chronic hyperglycemia (i.e. elevated blood glucose levels) and disturbances of carbohydrate, fat, and protein metabolism. These disturbances are usually the result of absolute or relative deficiencies in insulin secretion and/or action. DM is divided into four subgroups : insulin-dependent, non-insulin-dependent, malnutrition-related, and other types of diabetes mellitus associated with certain conditions and syndromes (1). This thesis is only concerned with the first subgroup: insulin-dependent diabetes mellitus (IDDM), which accounts for about 10% of the cases. In general, IDDM is known to affect 0.5% of the population of Western nations (2).

## 1. The clinical presentation of IDDM:

IDDM is defined by the presence of the classical clinical symptoms which include thirst, polyuria, wasting, and/or ketoacidosis and the necessity of insulin injection to control the hyperglycemia and to prevent the occurrence of ketoacidosis. If ketoacidosis is not treated, it can lead to diabetic coma and eventually death. The mean age for the onset of IDDM in diabetes-prone individuals is about 11 years (3). The disease is most common in children or young adults of northern European origin, while it has

a much lower incidence in their Oriental or native American counterparts (1).

## 2. The etiology and cause of IDDM:

Although the exact etiology and cause of IDDM are not known, it's now accepted that the disease results from a complex interaction between genetics and the environment which eventually causes the destruction of the pancreatic islet insulin-producing cells, the β-cells. The destruction is **selective** for the β-cells . There are several lines of evidence suggesting that the destruction is the result of an autoimmune process which is discussed below. Loss of the β-cells and the concomitant insulin deficiency lead to the observed severe hyperglycemia and ketoacidosis and its related symptoms as mentioned above.

## 3. The islets of Langerhans and β-cells :

The islets of Langerhans (22) (23) are clusters of endocrine tissue scattered throughout the exocrine pancreas of vertebrates higher in evolution than the bony fish. In the adult human, the islets comprise 1-2 % of the pancreatic mass (about 1 gm). However, the islet mass is dynamic, adjusting and adapting to meet the metabolic demands of the individual which vary depending on the size, age, and level of

activity of that individual. When the islets, or more specifically the insulin-producing β-cells, fail to meet those demands, diabetes occurs.

The pancreatic islet is made up of four types of cells : insulin-producing β-cells (60-80%), glucagon-producing α-cells (15-20%) or pancreatic polypeptide producing PP-cells (about 5%), and somatostatin-producing δ-cells (3%). The β-cells are polyhedral and heavily-granulated with 300nm-wide insulin secretory granules, where insulin maturation from proinsulin takes place. For example, it's estimated that each rat β-cell contains about 1,000 granules. The α- and δ-cells, on the other hand, are more columnar and often dendritic in shape, respectively. They are also smaller than the β-cells. The islets are organized in a nonrandom fashion where the core of the islet is β-cells surrounded by a continuous mantle of non-β-cells, 1-3 cells thick.

The islets are encased in a capsule which is made up of a single layer of fibroblasts and the collagen fibres laid down by these fibroblasts. The islets are highly vascularized and have a direct arteriolar blood supply. They are innervated by sympathetic fibers from the celiac ganglion and by parasympathetic fibres from the vagus nerve.

The main function of the islets of Langerhans, and their cells, is control of metabolic homeostasis. For example, the β-cells function as "fuel-sensors", adapting the rate of insulin secretion in response to

the variation in plasma levels of glucose and other substances such as amino acids, fatty acids, and ketone bodies. Insulin secretion is also stimulated by vagus nerve fibres and inhibited by sympathetic nerve fibres. In addition to nutrients and neural factors, β-cell function is influenced by other factors such as gastro-enteric and other hormones.

# II. IDDM as an autoimmune disorder

Is IDDM an autoimmune disorder? The answer to this question came from the results of various studies on diabetic humans and animal models. Contemporary research, using advanced cellular and molecular methodologies, strongly implicates the immune system in the development of IDDM. Before discussing the scientific evidence supporting IDDM as an autoimmune disease, it is important to note that most of the evidence was collected from studies utilizing animal models.

## 1. The need for animal models in IDDM:

Although data from human studies have been accumulating in the literature, IDDM remains refractory to cure or prevention. This is largely due to the complex nature of the disease, which appears to be

a multifactorial one depending on intricate interaction between the genetic make-up of the individual and its environment. Therefore, there is enormous challenge in studying the disease and devising therapies for it.

There are several factors that contribute to the difficulty inherent in studying IDDM. First, the genetic component of the disease is polygenetic (4), and as a result, studying the inheritance is more feasible when using animal models. Second, the sudden onset of disease does not allow investigation of the pathological events leading up to the clinical presentation of the disease. Third, the diseased organ (the pancreatic islets of Langerhans) is inaccessible by conventional methods for obtaining samples for investigation (i.e. biopsies). Fourth, new therapies cannot be readily tested unless their risks are minimal or negligible. This is because if IDDM occurs, it is not lethal and can be treated with insulin (albeit imperfectly in terms of eventual development of vascular and neurological complications). These factors have necessitated the development of animals models to study IDDM.

Several animal models have been developed to study IDDM (24). The most widely studied animal models for the study of the pathogenesis of β-cell destruction and subsequent IDDM have been the non-obese diabetic (NOD) mouse and the Bio-Breeding (BB) rat in which the disease occurs spontaneously, similar to humans (5). The literature is rich with data collected on the NOD mouse; this is largely due to the availability of immunological reagents to study mouse

cells and molecules, more than those available for the rat. Therefore, studies in NOD mice will be mentioned periodically in this thesis; however, the thesis will concentrate on the BB rat as an animal model for studying IDDM.

## 2 The scientific evidence for IDDM as an autoimmune disease:

There is abundant evidence that IDDM is a disease in which β-cells are destroyed by an autoimmune response that may be directed against particular β-cell components. This autoimmune response occurs in the appropriate genetic background, i.e. the predisposed individuals possess certain susceptibility alleles and lack other protection alleles which regulate the immune response. It is also important to note the role of the environment at this point. It is believed that environmental factors (microbial ?, dietary ?) may provide the initiating signal which induces an autoimmune response in genetically susceptible individuals. The susceptibility alleles will be discussed in detail below. Alternatively, environmental factors may avert or suppress an autoimmune response in a genetically susceptible individual (25).

There are direct and indirect factors that determine whether the disease is autoimmune. The genetic background of the individual and the damage caused by the immune response are important factors, but more important is the role played by key immune cells

(e.g. T-cells) and their ability to transfer disease. The following sections will discuss the scientific evidence of these aspects of autoimmunity in IDDM.

**a. IDDM is associated with certain MHC gene loci:**

The initiation of the autoimmune response depends on the presence of the appropriate susceptibility alleles and the absence of protection provided by other alleles. IDDM appears to be a familial disease, but the mode of inheritance is not yet fully understood. It is not Mendelian, and has defied comprehensive analysis or classification under any known genetic scheme. This could be due to the polygenetic nature of the disease. However, there seems to be a clear association between the disease and the presence of certain genes. The first and most notable of these genes is the major histo-compatibility (MHC) genes which are known in humans as leukocyte antigens (HLA).

HLA genes are present on chromosome 6 and encode cell surface proteins involved in antigen presentation to the T-cells. The structure, function and classification of the biological molecules have been discussed in detail (6). The role of HLA class I and class II molecules in the development of diabetes appears to be very complex and controversial. While it appeared initially that there was some association between IDDM and class I HLA-B8 and -B15 haplotypes, the disease was later found to correlate more strongly

with class II haplotypes : HLA-DR8, -DR1, -DR3, and -DR4 in order of increasing risk. On the other hand, HLA class II genes DR2 and DR5 were found to correlate with reduced risk of the disease.

The association of DR4 with the development of IDDM results from an amino acid occupying position 57 in the β-chain, which is encoded by the HLA-DQw3.2 gene (7). Some studies reported that the presence of aspartate (Asp) residue at this position conferred a strong protective effect among Caucasians. It was thought that the absence of Asp at this position results in the formation of defectively configured class II molecule which could facilitate the presentation of an autoantigen peptide fragment to the immune system. It may also be defective in the presentation of a self antigen during thymic ontogeny thereby resulting in loss of tolerance to this antigen in the periphery (i.e. defective negative selection). However, the protective role of DQbAsp57 became questionable when the studies were done using ethnic populations other than Caucasians. HLA-DQA1A*3 was found to be a susceptibility allele among Japanese and DR3/DRw9 in Chinese, whereas DR3/4 were not risks among Ashkenazi Jewish or Hispanic IDDM patients.

To explain the complexity of these immunogenetic associations, it has been suggested (8) that the relative affinity of the entire repertoire of available HLA class II molecules determines an individual's susceptibility to the development of IDDM. In other words, the individual is susceptible by the presence of a high-risk class II molecule and can be rendered low risk if other molecules

encoded by other class II genes have an even greater affinity for the diabetogenic peptide, thereby sequestering it from the immune system, or presenting the peptide better and thereby ensuring negative selection and tolerance induction.

Similar to humans, the role of MHC genetic influence has been reported in animal models. The protective role of DQbAsp57 was associated with IDDM in humans and one animal model, the popular NOD mouse, but it does not seem to play a role in the other genetically susceptible animal model, the BB rat. In the NOD mouse, diabetes is associated with a unique class II antigen, I-$A^{nod}$, and a complete absence of message, and consequently, protein expression of the I-E molecule (9). When these mice were genetically engineered to express a recombinant I-E gene, insulitis and diabetes did not develop, indicating the protective role of this molecule in preventing autoreactive cells from emerging (10) (11). Investigation of the genetic basis in the NOD mouse has also revived the importance of the role of the class I molecule which was found to play an important role in the development of self-tolerance and initiation of insulitis (12).

IDDM was also found to associate with the expression of other genes which encode proteins that are directly or indirectly involved in the development of the disease. The BB rat provides a good example of three non-MHC genes involved in the development of diabetes. This will be discussed thoroughly in the section dealing with the BB rat as a model for IDDM. With respect to the NOD mouse,

more than 12 genes have been reported to be associated with risk of developing IDDM (4).

**b. IDDM can be transferred by bone marrow cells :**

There are two reports in the literature for bone marrow transplantation in which the donor had IDDM and the recipient was normal. The transplantation resulted in the recipient acquiring IDDM. In the first transplantation, the recipient was a patient with severe aplastic anemia who received a bone marrow donated by his HLA-compatible sibling and acquired islet-cell antibodies (ICAs) 5 years after transplantation (13)(14). Similarly, the bone marrow in the second transplantation was donated by a histo-compatible brother with IDDM to a patient with acute lymphocytic leukemia. The recipient developed hypothyroidism and ICA-positive IDDM 3 years after the operation (14).

Although, the direct connection between the donated bone marrow lymphocytes and the development of IDDM has not been proven, it is likely that the production of ICAs and anti-thyroid antibodies in the pancreas and the thyroid were due to bone marrow cells from the donor, presumably lymphoid cells. In support of these reports, a world wide survey on bone marrow transplantations disclosed three other similar cases (15).

With respect to the ability of autoantibodies to transfer IDDM, a study (16) which reconstituted severe combined immunodeficient (SCID) mice with peripheral blood mononuclear cells from IDDM patients showed that the human B-cell-associated autoantibodies could be transferred to SCID mice and remained antigen sensitive. However, the autoantibodies alone were not sufficient to induce β-cell destruction. This indicates that the β-cell destruction relies more on T-cells. Extensive evidence supporting this comes from studies done in animal models which will be discussed in detail in the section dealing with the role of T-cells in IDDM development.

**c. IDDM recurs after normal pancreas or islets are transplanted into hosts with IDDM:**

Recurrence of IDDM after transplantations of normal islets into a diabetic patient can be considered the mirror image of the adoptive transfer of bone marrow or T-cells from a diabetic patients. In these transplantations (17), the pancreatic islet grafts were infiltrated with mononuclear cells, islet β-cells were destroyed and IDDM recurred in the recipients. This study provides strong evidence that the immune system of the recipient is still activated and capable of recognizing and destroying the β-cells presumably in response to an initiating signal from the newly-transplanted islets. However, the precise mechanism of this immune response is not yet clear.

**d. The disease can be prevented or slowed by deletion of T-cells or by the administration of immunosuppressive agents that target T-cells:**

IDDM can be prevented in NOD mice and BB rats by neonatal thymectomy, backcrossing to athymic animals, or administration of anti-T-cell antibodies. Immunosuppressive agents such as cyclosporine (CsA) and azathioprine plus steroids were also found to be effective in slowing and sometimes arresting the progression of IDDM in animal studies and also in humans. These agents are known to mainly suppress T-cells. The mechanism by which CsA suppresses T-cells is by inhibiting the production of the cytokine interleukin 2 (IL-2), which is required for the proliferation and activation of T-cells (18) (19).

**e. The disease is associated with humoral or T-cell mediated immune responses directed against the target organ:**

Since the destruction of the insulin-producing β-cells is thought to be immune-mediated, the presence of antibodies against the islet cell surface and islet intracellular contents is expected. It was first reported (20) (21) that islet-cell antibodies (ICAs) were detected in some sera of newly diagnosed IDDM patients. A subsequent and more comprehensive study (26) found them to be common in IDDM patients. The authors of this study reported the presence of ICAs in

the sera of 81% of the diabetic children surveyed as compared with only 3% in the control subjects (26). However, the methodological approach of these experiments was somewhat crude and resulted in the positive signal lacking specificity to the β-cells.

Antibodies against β-cell specific targets were also found. Serum antibodies against Insulin (IAA) were found in 50% of newly diagnosed diabetic patients prior to the onset of diabetes and insulin-therapy (27). In addition, an antibody (28) that recognized an islet protein (64kd) was found only in IDDM patients and not in non-diabetic subjects. This islet protein was subsequently identified to be an isoform of the gamma-aminobutyric acid-producing enzyme, glutamic acid decarboxylase (GAD), present in the cytoplasm of the islets. Serum antibodies to GAD were also found in IDDM animal models (29) (30).

Collectively, these studies indicate that there are humoral immune reactions against the islets, their products, or more specifically against some cytoplasmic constituents of the islets, during IDDM. The presence of antibodies against the intracellular components of the pancreatic β-cells might be expected, however, once they are destroyed. These antibodies do not provide evidence supporting a role for the antibodies in the initiation of β-cell destruction. Therefore, the discovery of antibodies directed against the surface of islet cells might shed some light on potential autoantibodies that might cause the initial damage to the islet cells. Such islet-cell-surface autoantibodies were discovered to be present

in human diabetic children and not in healthy ones (31). In addition, such autoantibodies were also discovered in animal models (see BB rat section). Nevertheless, islet-cell autoantibodies (cytoplasmic or surface) have not been capable of adoptively transferring IDDM whereas bone marrow or T-cells have. Therefore, autoantibodies are considered secondary "markers" of IDDM and are unlikely initiators of β-cell damage.

The literature documenting the central role played by T-cells in IDDM pathogenesis is extensive. In summary, the disease can be prevented in IDDM animal models like the BB rat and the NOD mouse by neonatal thymectomy, backcrossing to athymic animals, or administration of anti-T-cell antibodies (discussed in details in section III).

**3. The pathological effects of the immune response against the target organ:**

In addition to the presence of islet autoantibodies in the sera of prediabetic and diabetic individuals, the most pronounced effect of the autoimmune response is the inflammation of the islets, better known as insulitis. The term insulitis was first coined by Willy Gepts in 1965 (32). After initially describing the pathology of juvenile diabetes using the pancreas of a young diabetic patient, he continued the investigation of the disease in a much larger study. The

materials for his study consisted of fifty four pancreases from young diabetic patients in whom the onset of the disease had occurred prior to the age of thirty one, and twenty six pancreases from nondiabetic patients which served as control material.

The study showed that the islets of Langerhans in the diabetic patients were often large, i.e. inflamed. The peri- and intra-insular inflammatory infiltrates were observed in 70% of the cases. The proportion of insular tissue decreased in the diabetic patients. In addition, the number of pancreatic β-cells in the diabetic subjects was greatly reduced by 90% compared with the nondiabetic ones. The diabetic pancreatic β-cells showed cytological signs of intense secretory hyperactivity such as cellular and nuclear hypertrophy as well as an increase in the cytoplasmic ribonucleins. This is often interpreted as the attempt of the few remaining functional β-cells to compensate for the function of the destroyed ones and supply sufficient insulin to sustain glucose homeostasis. Insulitis is also found in the animal models for IDDM (discussed in detail later).

Subsequently, the cellular composition of the insulitis lesion in the human diabetic subject has been studied (33). In this study, the rare availability of a fresh human pancreas from a twelve-year-old girl recently diagnosed with IDDM, and who died in a ketoacidotic coma within 24 hours of the diagnosis, allowed the investigators to examine the nature of insulitis and the immunologic insult that selectively affected the β-cells. Using monoclonal antibodies that recognized all different leukocytes, it was determined that the

infiltrate was composed of mononuclear leukocytes, predominately T-cells. Also the majority of the T-cells in the infiltrate were of the cytotoxic/suppressor ($CD8^+$) phenotype. The T-helper/inducer ($CD4^+$) phenotype and natural killer (NK) cells were also observed in the infiltrate. In this study, macrophages or monocytes were not identified in the patient's pancreas. This can be due to the late timing of the tissue harvest as macrophages might play their role of antigen presentation early in the course of disease.

# III. The role of T-cells and their products in IDDM

## 1. Direct experimental evidence for the role of T-cells in IDDM:

The previous section dealt with the evidence supporting IDDM as an autoimmune disease. There was a strong indication from the evidence presented that IDDM is a T-cell-mediated autoimmune disease. The evidence that T-cells are central to the development of IDDM can be summarized along 5 experimental lines: thymectomy, adoptive transfer, immunosuppressive agent administration, administration of antibodies to T-cells, and detection of T-cells and their products in the lesions.

**a. Thymectomy :**

Evidence for the effect of thymectomy on the development of IDDM comes from animal model studies. Thymectomy prevents IDDM development in the BB rat, probably because autoreactive T-cells result from defective thymic ontogeny (34). Complete neonatal thymectomy of BB rats resulted in prevention of IDDM (from 27% to 3%) and reduction of insulitis. Incomplete thymectomy also significantly reduced the frequency of IDDM (to 9%). On the other hand, it was observed (35) that $CD4^+$ T-cells may play a protective role against the development of diabetes in thymectomized NOD mice.

**b. Adoptive transfer:**

In human studies (13) (14) (15), evidence from bone marrow transplantations showed that bone marrow cells played an important role in transfer of diabetes. The complementary observation that IDDM recurred after transplantations of normal islets into a diabetic patient also provided evidence for the role of immune memory cells in IDDM (41). In addition, passive transfer of Concanavalin-A-treated spleen cells from diabetic BB animals into young DP-BB rats resulted in the development of severe insulitis and diabetes (37). Furthermore, experimentally-induced diabetes in the DR-BB rat was adoptively transferred (38) to athymic nude rats (lacking B- and T-cells) using lymph node T-cells. Both $CD4^+$ and $CD8^+$ T-cells were required for efficient transfer of IDDM and IDDM was prevented when the transferred T-cells were mixed with known regulatory T-cells ($RT6^+$).

The $RT6^+$ system and its role in protection is discussed in detail below (see section VII.3.b).

The other popular IDDM model, the NOD mouse, has contributed more to the understanding of IDDM. With many studies on adoptive transfer using NOD, and variations thereof, it was shown that the disease was transferred using spleen cells from diabetic animals (39); and more successfully using islet-infiltrating lymphocytes from prediabetic NOD (40). Furthermore, the studies showed that both $CD4^+$ and $CD8^+$ T-cells were required for efficient transfer of IDDM and that $CD4^+$, and not $CD8^+$, T-cells play an essential role in syngeneic islet graft rejection (41).

**c. Cyclosporine (CsA) administration :**

Immunosuppressive drugs and corticosteriods were used in human and animal model studies to examine their effect on IDDM development. Most of these agents, especially cyclosporine (CsA), are known to interfere with T-cell function. In humans, CsA was found to increase the length and rate of remissions of IDDM in patients with recent onset IDDM (42). Treatment with CsA has also been successful in delaying and preventing the development of IDDM in the DP-BB rat when it was administered for a 10-day interval prior to the onset of disease (43). Histological examination from these studies indicated that the effect of CsA was specific to the insulitis lesion and did not affect the presence of autoimmune thyroiditis that usually accompanies insulitis. The authors speculated that the effect of CsA

was not seen in thyroiditis because the regulatory T cells that modulate it are different populations from those that modulate insulitis, and that a different dose may be needed to affect thyroiditis development. Therefore, the modulation of T-cells by CsA is specific to IDDM and occurs only in a small time window before the onset of disease.

**d. Antibodies to T-cells :**

A wealth of literature documenting the use of antibodies directed at T-cells in animal models is available. IDDM was prevented in the DP-BB rat with antiserum to rat lymphocytes (44). There are also several studies using monoclonal antibodies directed against specific T-cell subsets that led to the delay and/or prevention of diabetes (discussed in details below). Supporting the role of T-cells in disease development in animal models, various studies using antibodies against T-cells in the NOD mouse were reported to have the same preventive effect on the development of IDDM as in the BB rat (12).

**e. Detection of T-cells and their products in the lesions:**

The presence of T-cells in the insulitis lesion in human patients is documented in the literature despite the difficulty of obtaining the proper specimen for examination (33) (45). With respect to the BB rat, the cellular and temporal composition of the insulitis lesion has been characterized (46), and the products of the islet-infiltrating mononuclear leukocytes have been investigated in a few studies (47)

(48) (49) (50) and more thoroughly in this thesis. Several studies identified the phenotypes and products of islet-infiltrating mononuclear leukocytes in the NOD mouse (51).

## 2. T-cell subsets

T-cells are generally divided into two major groups: $CD4^+$ and $CD8^+$ (52). CD denotes a cluster designation molecule which is a cell-surface antigen that serves, in the case of CD4 and CD8, as co-receptor for the T-cell receptor (TCR). $CD4^+$ T-cells, sometimes referred to as "helper/inducer T-cells", proliferate in response to antigen and stimulate other cells to become cytotoxic, and induce B-cells to secrete antibodies. $CD8^+$ T-cells, on the other hand, are capable of both direct cellular cytotoxicity and immunosuppressive activities. Classical immunological concept states that β-cell destruction in IDDM results from killing by antigen-specific $CD8^+$ T-cells that have been activated by $CD4^+$ T-cells. However, other models are also likely to explain the mechanism by which the β-cells are destroyed (discussed in detail in section V). In any event, both $CD4^+$ and $CD8^+$ T-cells are required for the development of disease. Depletion of either T-cell subset protects the autoimmune diabetes-prone animal from the development of IDDM, with varying degrees depending on the experimental system used.

**a. Role of $CD8^+$ T-cells in IDDM:**

The role of $CD8^+$ T-cells in the development of IDDM was initially thought to be the direct killing of β-cells according to the classical concept. $CD8^+$ T-cells depletion in the diabetes-prone BB rat protected against IDDM (53). Furthermore, adoptive transfer of IDDM in the same animal model required $CD8^+$ T-cells to be present in the donor cell population (54). Recipients that were treated with monoclonal antibodies to deplete $CD8^+$ T-cells specifically did not develop IDDM (0%).

In the NOD mouse, the role of $CD8^+$ T-cells was also investigated further, and it seems that $CD8^+$ T-cells play a complementary role to $CD4^+$ T-cells. Although, the adoptive transfer (39) of spleen $CD8^+$ T-cells alone from NOD mice into NOD/*scid* mice did not result in the development of IDDM, the transfer of unfractionated spleen T-cells or spleen $CD4^+$ T-cells alone did cause IDDM. The treatment of the latter recipients with $CD8^+$ monoclonal antibodies reduced, but did not prevent, the incidence of diabetes. The antibodies in this case were directed at $CD8^+$ T-cells which presumably developed from the donor $CD4^+$ T-cell clones during or after the transfer.

The cytotoxic role of $CD8^+$ T-cells in the destruction of pancreatic β-cells in NOD mice was investigated (56). The transfer of these islet-specific $CD8^+$ T-cells into irradiated NOD mice caused insulitis and diabetes but only in the presence of $CD4^+$ T-cell splenocytes, whereas the depletion of $CD8^+$ T-cells resulted in

prevention of diabetes. Furthermore, electron microscopic studies revealed that islet-specific $CD8^+$ T-cells showed pseudopodlike protrusions into the β-cells but not α-cells or δ-cells. On the other hand, islet-specific $CD4^+$ T-cells attached closely to islet cells but did not destroy them (57).

More recently, it has been shown (58) that $CD8^+$ T-cells play an important role in the initiation of IDDM. The depletion of $CD8^+$ T-cells from the NOD mouse by administration of anti-$CD8^+$ T-cell monoclonal antibody during a discrete age window (between 2 and 5 weeks from birth), not only prevented diabetes but also insulitis. The disappearance of insulitis from these animals strongly suggests that $CD8^+$ T-cells play an important role in initiation of the disease. In support of these results, insulitis and IDDM did not develop (59) in class I MHC deficient ($\beta 2m^{null}$) mice, indicating the autoimmune IDDM in the NOD mice is initiated by MHC class I-dependent T-cell response (i.e. $CD8^+$ T-cells).

**b. The role of $CD4^+$ T-cells:**

The role played by $CD4^+$ T-cells in the development of IDDM is more pivotal than $CD8^+$ T-cells. Studies done in NOD mice indicate that the disease is the result of $CD4^+$ T-cell-dependent inflammatory tissue damage. When NOD spleen $CD4^+$ T-cells were adoptively transferred into NOD/*scid* recipients, IDDM ensued with a high frequency (~90%) (39). In addition, the administration of anti-$CD4^+$ T-

cell monoclonal antibodies to both the NOD mouse and BB rat animal models halted the progression from insulitis to overt diabetes in the former (12) and nearly completely prevented insulitis, if administered sufficiently early, in the latter (60). Furthermore, islet transplantation studies in NOD mice showed that recurrent disease depended on $CD4^+$ T-cells, and not $CD8^+$ T-cells, and that depletion of the $CD4^+$ T-cell subset facilitated syngeneic islet graft acceptance (7).

The role of $CD4^+$ T-cells in the development of IDDM does not appear to be simple. Some studies reported that cotransfer of $CD4^+$ T-cells from prediabetic female mice was able to delay and reduce the incidence of diabetes adoptively transferred by spleen cells from female diabetic donors into NOD/*scid* recipients (40). It was also reported that administration of anti-$CD4^+$ T-cell antibodies to male NOD mice, which are less diabetes-prone than female NOD mice, rendered the male mice susceptible to diabetes transfer by diabetogenic spleen cells (35). These results suggests that "diabetes-resistant" animals possess a regulatory $CD4^+$ T-cell subset that provides protection against the development of diabetes. The regulatory fraction appears to exist in the diabetes-prone animals as well, but is overcome by the autoreactive T-cells during the course of diabetes. Therefore, the role that $CD4^+$ T-cells play is now considered a pivotal one directing the immune response toward destruction of the β-cells in the diabetes-prone animals, or toward protection in the diabetes-resistant animals. In fact, the current thinking regarding the mechanism of IDDM development centres around the role of $CD4^+$ T-cells in controlling destruction and/or protection of the β-cells.

There is also evidence that islet-specific $CD4^+$ T-cell clones can destroy the β-cells (61).

$CD4^+$ T-cells are made up of a few subsets that are still under investigation and further fractionation. These subgroups are defined based on their surface markers and secretory products known as cytokines.

### 3. Cytokines and $CD4^+$ T-cell subgroups:

The term 'cytokine' refers to a group of protein cell regulators, variously called lymphokines, monokines, interleukins and interferons. Cytokines are small proteins that are synthesized and secreted by activated immune and non-immune cells to act as inter-cellular communication messages, and consequently they control local and systemic events of immune and inflammatory responses (62). Cytokines are fast acting with a relatively short half-life in the circulation (36) (55) (63), but are capable of influencing many physiological processes in the body because of their auto-, para-, and sometimes endocrine modes of action (64). Cytokines are also highly potent; one activated cell can produce sufficient cytokine protein to activate 1000-10,000 other cells. This is due to the unique biology of cytokines in which the cell source, despite its low frequency amongst cells, is capable of producing a large amount of cytokine protein upon stimulation. In addition, the cytokine receptors have high affinity for their cytokine ligands (65).

More than 30 immunologically active cytokines exist. They are usually grouped as interleukins, interferons, tumor necrosis factors, and colony-stimulating factors. The description of the structure and function of each cytokine is beyond the scope of this thesis; however, they will be briefly mentioned throughout the thesis when relevant or necessary. Also, section IV.7 below contains brief discussion of the induction and function of the relevant cytokines in this thesis.

The interpretation of cytokine action is complicated because of their redundancy and ambiguity. The former refers to structurally dissimilar cytokines that have an overlapping spectrum of actions, while the latter is due to multiple target cells and actions. However, the action of cytokines is very essential to the function of antigen-activated T-cells, termed T-helper cells (Th), because they mediate both cellular and humoral immune responses.

The discrete Th subsets were originally described (66) in mouse immune responses; however, they were subsequently also found in humans. Recent immunological studies on the rat indicate that the discrete Th subsets may also exist. Initially the discrete Th T-cell subsets fell into two classes: Th1 which is involved in cell mediated inflammatory reactions (e.g. delayed-hypersensitivity, DTH) and Th2 which encourages antibody production and enhancing eosinophils' proliferation and functions. The Th1 and Th2 correlate with distinctive cytokine production patterns: Th1 is usually associated with the production of interferon gamma (IFNγ), interleukin 2 (IL-2),

and Tumor necrosis factor beta (TNFβ), whereas Th2 correlates with IL-4, IL-5, and IL-10 production. The two subsets were found to be mutually inhibitory (67).

Further investigation revealed that the discrete subsets should be expanded to include Th0 which secretes a cytokine pattern similar to both Th1 and Th2 combined (IFNγ, IL-2, and IL-4) (68) (69), and Th3 which is known to secrete transforming growth factor beta (TGFβ) (70). Despite the fact that more Th subsets are being discovered, cytokine expression from these subsets appears nonrandom. In addition, although the work done to characterize these subsets used *in vitro* T-cell clones, *in vivo* immune responses also show dichotomy between IFNγ/IL-2 and IL-4/IL-5 cytokine patterns (71) (81) (82).

The differentiation of the different Th subsets from a common precursor cell depends on a number of important factors (summarized from the reference (72)) :

i. Antigen dose : At low and high concentration of antigen (*in vivo* ), Th1 differentiation occurs, whereas Th2 differentiation takes place at medium antigen concentration.

ii. Antigen-presenting cell (APC) types: Macrophage/monocytes and dendritic cells secrete IL-12 which induces Th1 differentiation and B-cells produce IL-4 which stimulates Th2 differentiation.

iii. Co-stimulatory molecules: The costimulation provided by B7 during the MHC antigen-presentation to the TCR influences differentiation of the Th subsets. Involvement of the B7.1 molecule in costimulation directs differentiation towards Th1, while B7.2 leads to Th2 differentiation.

iv. Neighbouring cytokines: The presence of certain cytokines in the proximity of differentiating Th cells dictates the direction of differentiation. As mentioned above, the presence of IL-12 and IL-4 stimulate Th1 and Th2 differentiation, respectively. Such cytokines could be released by APCs or other non-immune cells (e.g. endothelial cells) and as a result are present in the local environment of the Th T-cells.

With respect to $CD8^+$ T-cell differentiation, it was observed that they often secrete a Th1-like cytokine pattern, but now there is increasing evidence that a Th2-like $CD8^+$ T-cell differentiation also exists (73). The two patterns are now known as Tc1 and Tc2, respectively (156). IFNγ and IL-12 encourage the differentiation of $CD8^+$ precursors into Th1 or Tc1 cells which produce IFNγ and IL-2. IL-4, on the other hand, induces the generation of Th2 or Tc2 cells, which produce IL-4, IL-5, and IL-10.

# IV. Cytokines in IDDM

The classification of the Th T-cell subsets as discussed above came from phenotyping T-cell clones *in vitro*. The *in vivo* relevance of such Th T-cell subsets was proven when they were documented to control immune responses to infections in animals (71). For example, mice strains (e.g. C57bl/6, B10.D2, and C3H/HeN) that are known as genetically prone to mount Th1 responses to infection are capable of resisting *Leishmania major* infection, whereas mice biased toward Th2 response (e.g. BALB/c) cannot control the infection. By the same token, T-cell subsets could be responsible for induction and control of immune responses in autoimmune diseases. The role of Th1 and Th2 cells in organ-specific autoimmune diseases is currently under intense investigation. Two such diseases present a scenario where Th1 cells may be pathogenetic while Th2 may be protective: EAE, a model for multiple sclerosis, and IDDM.

T-cell subsets appear to play a role in the pathogenesis of IDDM. Adoptive transfer (74) of $CD4^+$ T-cell lines into neonatal mice showed that IFNγ-secreting Th1 cells induced overt diabetes while IL-4-secreting Th2 cells resulted only in a nondestructive peri-islet insulitis. The donor $CD4^+$ T-cell lines were generated from the spleens of diabetic NOD mice against crude membrane preparations derived from a rat insulinoma. In another study (75), the proposition that Th1 cells promote IDDM but Th2 cells do not, was tested using established cultures of $CD4^+$ Th1 and Th2 cells from a diabetic NOD mouse. The T-cells were specific for an islet antigen. The adoptive

transfer of the $CD4^+$ Th1 subset in neonatal NOD mice actively promoted disease, whereas the $CD4^+$ Th2 subset transfer resulted in the cells infiltrating the islets but not provoking disease. Co-transfer of Th2 cells with Th1 cells, however, did not prevent the Th1 cells transferring IDDM. In other studies, male NOD mice with low incidence of diabetes had a Th2 bias, while the female NOD mice with high incidence showed a Th1 bias in terms of the ratio of IFNγ/IL-4 mRNA expressed in pancreatic islet-infiltrating leukocytes (76).

As mentioned above, the Th lymphocyte subsets are defined by the cytokines they produce. The Th1 T-cells secrete IFNγ, IL-2, and TNFβ, while Th2 secrete IL-4, IL-5, and IL-10. However, it is important to recognize that most cytokines are produced by more than one cell type. Therefore, the nomenclature now generally recognized defines the cytokine on the basis of the response it modulates instead of the cell type that produces the cytokine. Thus, the Th1 and Th2 cytokines are also termed as type 1 and 2 cytokines, respectively, to include other cell types that might produce them such as $CD8^+$ T-cells (Tc1 and Tc2).

The role of cytokines in IDDM was recently reviewed (77). Three general approaches have been used to study the roles of cytokines in the pathogenesis of IDDM: 1) correlation studies of cytokines expressed in the pancreatic islets or in the periphery in relation to diabetes development, 2) cytokine augmentation studies in which cytokines are added to islets *in vitro*, hyperexpressed *in vivo* (e.g. transgenic expression) or administered systemically *in vivo*, and 3)

cytokine deficiency studies in which cytokine genes (i.e. knock-out animals) or proteins (by administration of cytokine-specific monoclonal antibodies) are experimentally deleted.

**1. Effect of cytokines on islet cells and insulin secretion *in vitro* :**

Addition of the type 1 cytokine IFNγ, or other cytokines like TNFα/β or IL-1, to pancreatic islet *in vitro* (in picomolar concentrations) was found to inhibit insulin synthesis and secretion. However, the islet cells were able to recover after the cytokine is removed. This is known as cytostatic interaction with the β-cells. On the other hand, when these cytokines are added to islets in combination, they became cytocidal. In other words, they destroyed the β-cells of both purified rodent and human islets *in vitro* (78).

**2. Expression in spontaneous or recurrent disease:**

Type 1 cytokines (IFNγ, IL-2, and TNFα) are elevated in the serum of recently diagnosed diabetic patients (79) (80), whereas type 2 cytokines, IL-4 and IL-10 are not (79). IL-12 expression also was elevated in the sera of high risk IDDM first degree relatives (79). In the NOD mouse, studies (82) (83) (84) of cytokine gene expression revealed that IFNγ in the insulitis lesion was associated with destruction of the β-cells, and expression of IL-4 and IL-10 correlated with protection of the syngeneic islet grafts. In addition, cytokines like IL-12 (86) (87)

and IL-18 (IGIF) (88), that are produced by macrophages and other APCs and that drive the production of type 1 cytokine response, have been also found to be expressed in the pancreas or islets with β-cell destructive insulitis.

Although not as investigated as much as in the NOD mouse, expression of type 1 cytokines in the diabetes-prone BB rat has also been reported (47) (49) (50). This thesis will also deal with the expression of cytokines in the BB rat. However, it's important to note that the mere presence of a particular cytokine in the lesion does not identify its particular role in the development of disease.

### 3. Transgenic expression of cytokines:

Expression of Th1 cytokine transgenes reveal the local pro-inflammatory effects of such cytokines in the islets. IFNγ transgenics have shown that pancreatic expression of this important type 1 cytokine is capable of inducing insulitis and diabetes in nondiabetes-prone mice (89). In addition, the lymphocytes recruited to the islets by the ectopic expression of IFNγ were also found to be cytotoxic to normal islets *in vitro* and engrafted histocompatible islets *in vivo* (90). Transgenic expression of IL-2 also resulted in the acceleration of diabetes in the NOD mice (91). Expression of TNF transgenes in the islets, however, showed that these cytokines could lead to insulitis but not diabetes even in the diabetes-prone mice (92).

Transgenic expression of the Th2 cytokine IL-4 in the diabetes-prone female NOD mouse, on the other hand, was effective in the prevention of insulitis and diabetes (93). Furthermore, the expression of IL-4 in the pancreas alone was effective in the induction of functional tolerance to the islet antigens indicated by the inability to reject syngeneic pancreatic islets in the prediabetic recipient NOD mice and the failure of diabetogenic spleen cells to induce diabetes in NOD-IL-4 recipients. IL-10 transgenics, however, showed that local IL-10 production was unable to inhibit immune-mediated tissue destruction in IL-10 and LCMV double transgenic mice (94), rather IL-10 acted as a pro-inflammatory cytokine in non-diabetes-prone mice (95). Furthermore, IL-10 was found to result in destruction of the β-cells in mice with a diabetes-permissive genetic background, namely the NOD mouse (96) (97).

**4. Systemic administration of cytokines :**

Systemic administrations of type 2 cytokines, IL-4 (98) and IL-10 (99), to diabetes-prone NOD mice have resulted in the prevention of diabetes development. Furthermore, systemic administration of a long-lived IL-10/Fc fusion protein to NOD female mice from 5 to 25 weeks of age significantly reduced insulitis to small peri-islet aggregates of mononuclear leukocytes and completely prevented the development of diabetes as long as long as the treatment was administered (100). It was also reported that the treatment reduced the percentages of islet-associated leukocytes secreting type 1 cytokines (IFNγ and IL-2) and TNFα and increased the percentages of

those secreting type 2 cytokines (IL-4 and IL-10). The treatment was also able to generate splenic cells that prevented the adoptive transfer of diabetes by acutely-diabetic spleen cells transferred into irradiated NOD mice.

TNFα administration has contradictory effects on the incidence of diabetes in diabetes-prone animals: it increased the incidence of diabetes if administered to NOD mice early in life but protected against the development of diabetes when the treatment started after 4 weeks of age (101). The administration of other cytokines, like IL-1, has yielded inconclusive results with respect to their effects on diabetes development. Systemic administration of large daily doses of IL-12, a cytokine known to induce Th1 differentiation, accelerated the development of diabetes in NOD mice (102). This acceleration was also associated with increased IFNγ and decreased IL-4 production by islet-infiltrating lymphocytes. On the other hand, administration of low doses, once a week, suppressed the development of diabetes (103). Therefore, the effects of systemic administration of cytokines on the incidence of diabetes depends on the dose, frequency, timing of administration, and the diabetes-prone animal studied. Also, it is important to note that a given cytokine may affect the production and action of other cytokines through an action on neuro-immunological circuits. For example, TNFα and IL-1 can affect the hypothalamic-pituitary axis leading to secretion of adrenocorticotropic hormones, and consequently adrenal corticosteroids which have a general suppressive effect on the inflammatory immune responses.

## 5. Cytokine gene "knock-out" animals in diabetes:

The most important cytokine gene knock-out (GKO) experiment reported to date has been the IFNγ GKO NOD mouse, which showed that IFNγ is <u>not</u> essential for β-cell destruction and the development of diabetes, i.e. diabetes onset was delayed but not prevented in the IFNγ GKO NOD mice (104). Interestingly, however, IL-4, as well as IFNγ expression was deleted in the IFNγ GKO NOD mouse. In contrast to persistence of diabetes development after abrogation of the IFNγ gene, mutation of the gene encoding the IFNγ receptor has recently been reported to markedly inhibit insulitis and completely prevent diabetes development (105). The difference between the effect of the deletion of the cytokine itself (IFNγ) and its receptor (IFNγ receptor) on the diabetes development in the two studies mentioned (104) (105) is interesting, since they were both expected to have the same effect. The difference may be due to technical differences (i.e. different studies involving different laboratories and investigators). Alternatively the difference may be due to functional cytokine functional redundancy where another cytokine may bind to the IFNγ receptor and mediate the same effects as IFNγ in its absence, whereas in the case of IFNγ-receptor deletion the function is completely abrogated. The effect of IL-$12_{p40}$ gene-targeted deletion in the development of diabetes is currently being investigated (106).

**6. Cytokine antibody administration :**

Studies involving administration of anti-cytokine antibody to deplete the cytokine from the circulation have also supported the role of type 1 cytokines (IFNγ, IL-2, and IL-12) in the development of diabetes. Administration of anti-IFNγ monoclonal antibody to both NOD mice and BB rats (107) led to protection against diabetes (108) (109). Administration of soluble IFNγ receptor also resulted in suppression of diabetes in the same animal model, the NOD mouse (110). In addition, administration of anti-IL-2 receptor monoclonal antibody also resulted in suppression of diabetes in the same animal model (111). The administration of anti-IL-$12_{p40}$ monoclonal antibody was also reported to decrease the incidence of spontaneous diabetes in NOD mice (106).

Alternative approaches were also employed to test the effect of interference with cytokine function by the administration of cytokine antagonists. It was recently reported (112) that the treatment of NOD mice with the homodimer IL-$12(p40)_2$, an antagonist to the natural IL-$12_{p35/40}$ heterodimer, prevented the development of cyclophosphamide-induced IDDM. The treatment with this antagonist also resulted in decreased IFNγ gene expression, and decreased the ratio of IFNγ/IL-10 mRNA levels, as well as suppressed the expression of inducible nitric oxide synthase.

Collectively, these lines of evidence support the role of type 1 cytokines in the promotion of IDDM, especially IFNγ. On the other

hand, the type 2 cytokine, IL-4, protects against the development of IDDM. However, the findings for TNFα and IL-10 appear to contradict the expectation that they are pro-inflammatory and suppressive cytokines, respectively. In fact, several pieces of evidence suggest that the roles that TNFα and IL-10 play depend on their time of expression in relation to the development of the autoimmune response to islet β-cells and the site of cytokine expression (intra- or extra-islet).

## 7. Review of important cytokines studied in this thesis:

The follow is a brief review of the basic information available regarding some of the important cytokines studied in this thesis:

### a. IFNγ :

The biologically active IFNγ is 34-kD homodimer (231) encoded in both human and mouse by a single copy gene on chromosome 12 (232) and 10 (233) , respectively. The gene consists of four exons and three introns, and the overall homology between human and murine IFNγ is 65% for nucleotide sequence and 40% for protein sequence. The gene is induced in T-cells (CD4 (234) and CD8(73)) and NK cells (235). The major inducer of IFNγ production by T-cells is cross-linking of TCR (236). In NK-cells, the IFNγ gene is induced by macrophage-derived cytokines like TNFα and IL-12 (237).

IFNγ interacts with a specific cell receptor that is ubiquitously, but not uniformly, expressed on all nucleated cells (238) at modest level (i.e. 200-25000 sites/cell) (239). The signal transduction pathway includes two important protein families Janus kinases (JAKs) and signal transducers and activators of transcription (STATs)(240).

IFNγ is capable of the induction of over 200 genes (241). The main function of these genes is to mount a strong immune response. IFNγ autoregulates (242) and synergizes with Type I interferons (α and β) (241). IFNγ enhances expression of interferon regulatory factors (IRFs) which lead to the induction of genes such as the iNOS gene (243).

IFNγ also has strong immunoregulatory role. It plays an important role in Th subset determination. Th1 secretes IFNγ (66) which propagates Th1 function through the reciprocal induction of IFNγ and IL-12 secreted by macrophages and neutrophils. IL-12 directly induces IFNγ gene transcription and secretion of IFNγ by NK cells (244). Therefore this positive feedback loop results in the propagation of Th1 cellular subtype. Conversely, IFNγ has a negative effect on Th2 (66). IFNγ downregulates IL-4 receptor β-chain expression in Th2 cells resulting in cellular desensitization. Also, IFNγ is the main switch factor regulating IgG2a (245) in the mouse but plays a minor role in regulating IgG3 switching (246).

**b. IL-2 :**

IL-2 was first discovered as in 1975 as a growth-promoting activity for bone marrow-derived T-cells (247). Since then, the activities of IL-2 have been discovered to include many more direct effects on B-cells, lymphokine-activated killer (LAK) cells, monocytes, macrophages, and oligodendrocytes.

IL-2 is a 15.5 kD protein secreted by activated T cells. The human IL-2 gene was mapped to chromosome 4q bands 26-28 (248). The inducible expression of this gene is controlled mainly at the transcriptional level through a 5' enhancer element where several functional *cis* -acting regulatory sequences, such as NFAT-1, NF-κB, AP-1, and octamer proteins, are known to bind (249). IL-2 gene expression is also controlled at a post-transcriptional level involving instability of IL-2 mRNA, apparently mediated through AU-rich sequence motifs which are present in the 3'-untranslated region of the IL-2 mRNA(250) (see TNFα below).

An overview of the signal transductional pathways involved in the induction of IL-2 gene transcription is covered in the literature (251). The induction of IL-2 gene transcription appears to be directly related to the interaction of the T-cell with the APC. This interaction involves not only the binding of the TCR+CD3 complex with the MHC+Ag complex, but also a number of receptor and adhesion molecules such as CD4, CD8, CD45, and CD28. Intracellular domains of CD3, CD4 (or CD8), and CD45 are involved in transducing signals

through $P59^{fyn}$ and $P56^{lck}$, and intracellaur phosphotyrosine phosphotases (PTPases), which eventually help translocate a variety of transcription factors into the nucleus where they bind to 5' enhancer element and increase transcription. CD28 intracellular domains are thought to transduce a signal resulting in prolonging the half-life of IL-2 mRNA.

The IL-2 receptor (252) is composed of three subunits α, β, and γ. The subunits α and β are members of the cytokine receptor family but γ is not. The IL-2 receptor may be present in three forms : the low affinity monomeric IL-2α, the intermediate affinity dimeric IL-2αβ, and the high affinity trimeric IL-2αβγ form. Signalling is usually through the long cytoplasmic domain of the β chain with src-family tyrosine kinases.

**c. IL-12:**

IL-12 was originally identified as cytotoxic lymphocyte maturation factor (CLMF) (253) or natural killer stimulatory factor (NKSF) (254) since it synergized with IL-2 to enhance the generation of LAK cell activity and enhanced the lytic activity as well IFNγ secretion from NK cells. IL-12 is a 75 kD heterodimeric cytokine consisting of two disulphide-linked subunits (255). The two subunits, p35 and p40, are encoded by two distinct genes which must be coexpressed in the same cell for the production of a biologically active protein. Chromosomal location of the p35 and p40 genes was

determined using DNA from human-rodent hybrid. The gene for p35 mapped to 3p12-3q13.2, and the gene for p40 mapped to 5q31-q33 (256). The degree of homology between human and murine cDNA sequences was found to be 60% for p35 and 70% for p40 (257).

IL-12p70 is produced by macrophages, dendritic cells, and neutrophils (255). IL-12 production can be induced by interaction with activated T-cells which provide co-stimulatory signal through molecules such as the CD40 ligand.

The major cellular targets for IL-12 are T-cells, NK cells, and B-cells at different stages of the immune response (255). The main effect of IL-12 appears to be the induction of IFNγ expression, which in combination with IL-12, leads to differentiation of Th cellular subsets into Th1 response (237).

The IL-12 receptor is composed of two chains, β1 and β2, which are expressed mainly by activated T-cells and NK cells. IL-12Rβ1 is expressed by both Th1 and Th2 cells (258), whereas IL-12Rβ2 is expressed in only Th1 cells thus accounting for Th2 inability to respond to IL-12 (259). The β1 chain is thought to interact with Janus-family kinase 2 (JAK2) and β2 with tyrosine kinases 2 (TYK2) and with signal transducers and activators of transcription 3 (STAT3) and STAT4 (260).

**d. IL-4:**

IL-4 (261) was first identified for its ability to induce activated mouse B cells to proliferate and secrete IgG1. The cellular sources of IL-4 include T-cells and basophil/mast cells. Studies (66) with murine helper $CD4^+$ T-cells showed that Th2 secrete IL-4, among other cytokines, in response to IL-4 secreted by mast cells. IL-4 is produced initially by mast cells activated by cross-linking the FcεR I and FcεRII and induces the differentiation into Th2 cells *in vivo* and *in vitro* (262).

The secreted murine protein of IL-4 is 120 amino acids (aa), while the human form is 129 aa (263). The chromosomal location (264) for the human IL-4 gene was mapped to 5q23-31, whereas the murine counterpart was located on chromosome 11.

IL-4 increases the volume of resting B-cells and induces their homotypic aggregation (265). IL-4 also induces hyperexpression of MHC class II antigens on murine B cells as well as the expression of CD23 (266), CD40 (267), BB1/B7 (268), and surface IgM (269).

**e. IL-10:**

IL-10 was originally discovered while searching for a cytokine produced by Th2 cells that could inhibit cytokine production by Th1 (270). Such a cytokine was discovered and named cytokine synthesis

inhibitory factor (CSIF) (271), then later renamed IL-10. It is also secreted by macrophages (272) and other types of cells including B-cells (273).

The mouse IL-10 is a non-disulphide-linked homodimer of approximately 35 kD (270). The monomer chains correspond to molecular masses of 17 and 21 kD. Both human and mouse IL-10 genes are found on chromosome 1 (274). The gene spreads over 1.5 kb containing 5 exons with upstream regulatory sequences that are shared among cytokines. The degree of homology between human and murine sequence is 90%.

IL-10 inhibits cytokine production by macrophages, NK cells, and Th1 cells (275). It also inhibits nitric oxide (NO) production and antigen presentation to Th1 cells by macrophages. On the other hand, it enhances B-cell proliferation, antibody secretion, and MHC class II expression.. It also enhances proliferation and protease expression by mast cells.

**f. TNFα:**

TNFα (276), also known as cachectin, is a 17 kD protein produced mainly by macrophages. TNFα was first identified for its potent toxicity and implicated as a lethal mediator of acute and chronic infection. Biosynthesis of TNFα is highly regulated at different levels.

The gene for TNFα is located on chromosome 6 near the HLA locus (human) and chromosome 17 (mouse) (277). Stimulation of macrophages with bacterial lipopolysaccharides (LPS, endotoxin) is a potent inducer of TNFα; it results in a 3-fold increase in TNFα transcription level. The transcription of TNFα is subject to regulation by sequences that lie upstream of the TATA box (278). These sequences are similar to the nuclear factor *k*-B enhancers, to the 'Y'-box promoter, to a cAMP-responsive element, and to the *jun*/AP-1 binding site.

The TNFα-gene product is also subject to regulation at the post-translational level via a conserved consensus octamer (UUAUUUAU) present in the 3' untranslated region of the mRNA for TNFα and other cytokines (250). This sequence confers a destabilizing influence and shortens the half-life of mRNA. Another regulatory region is a translational depression at the 3' untranslated region (279). This region results in acceleration of TNFα mRNA translation. While LPS is able to stimulate the production of TNFα, the biosynthesis of TNFα is suppressed by a number of factors including protoglandins ($PGE_2$), cAMP, activators of PKC, pentoxyphylline, and cyclosporine A (276).

TNFα has a variety of effects on a number of systems in the body including the renal, metabolic, gastrointestinal, cardiovascular, and central nervous systems (276). Discussion of TNFα on these systems is beyond the scope of this thesis. However, TNFα also affects the immune system in a variety of ways. In fact, investigation of the effects of TNFα on the different functional

aspects of immunity is a wide field of research currently studied by many labs. TNFα interacts with two type of receptors, p75 and p55, to cause an array of responses from killing by apoptosis to up-regulation of MHC class I molecules on the target cells (280).

# V. Immunological effector mechanisms for β-cell destruction

## 1. Possible mechanisms for β-cell destruction :

The previous sections have dealt with the role of T-cells in controlling the development of IDDM. This section will deal with the possible mechanisms by which the T-cells could directly destroy the target, the β-cells. There are four possible ways that T-cell can inflict a destructive effect on the pancreatic islet β-cells. These are described below.

### a. Cytotoxic cytokines :

As mentioned above, the presence of IFNγ, TNFα/β, and IL-1 cytokines proved to be harmful to β-cells and their function *in vitro*. IFNγ is usually produced by activated T-cells ($CD4^+$ or $CD8^+$ T-cells), while TNFα/β, and IL-1 are known to be secreted by activated

macrophages. These cytokines have all been detected in islets infiltrated by mononuclear leukocytes in NOD mice and BB rats.

**b. Cytotoxic free radical production by activated macrophages :**

IFNγ-activated macrophages in the insulitis lesion can also produce free radicals which are known to harm the β-cells. It was found (113) that macrophages in the islets of NOD mice expressed inducible nitric oxide synthase (iNOS) and peroxynitrite (the reaction product of nitric oxide and superoxide), in association with islet β-cell destruction. Also, β-cells themselves expressed iNOS and peroxynitrite in association with progressing insulitis and expression of IFNγ and IL-1 (114) (115).

While the first two cytotoxic mechanisms (described above) are not specific to the β-cells, the third and fourth mechanisms (described below) depend on specific recognition and direct contact of the target, the β-cells, by the leukocytes in the insulitis lesion.

**c. "Kiss of death", *Fas* and *FasL* system (receptor-mediated cytotoxicity) :**

This system is known to be involved in apoptosis (i.e. programmed cell death) (116) (159). Apoptosis has been suggested as the mechanism by which β-cells are destroyed during the development of diabetes (117). This suggested that Fas-and-FasL system may be involved in

diabetes development. The role of the Fas-and-FasL system in IDDM is currently under intense investigation. Fas/FasL-mediated mechanisms of β-cell killing have been investigated using FasL-transgenic NOD mice in which FasL was selectively expressed on the surface of islet β-cells (118). The authors found that FasL expression usually accelerated diabetes development in the NOD mice, and this was associated with Fas expression on the islet β-cells. In another approach, the authors crossed NOD mice with $MRL^{lpr/lpr}$ mice, which lack Fas expression, and they found that these Fas-negative $NOD^{lpr/lpr}$ mice did not develop diabetes and were resistant to adoptive transfer of diabetes by an islet-reactive $CD8^+$ T-cell clone. This suggested that Fas expression by islet β-cells may be essential for the islet β-cell destruction. In other words, the destruction process of the islet β-cells is not simply a function of increased islet-reactive $CD8^+$ T-cells attacking the islet β-cells, but rather a function of the specific expression of Fas by the islet β-cells.

**d. Secretory granule-mediated cytotoxicity :**

Cytolytic T lymphocytes (CTL) and NK cells can lyse their target cells directly utilizing a mechanism that requires transient but close contact between the killer and target cells. The "cytocide" takes a few minutes to occur and it can be resolved into three steps (119). First, the effector cells specifically recognize the appropriate target cell and bind to it due to the function of TCR and/or coreceptor molecules (e.g. CD8). This step is $Mg^{2+}$-dependent. Second, the delivery of the lethal hit, which is a $Ca^{2+}$- and temperature-

dependent process involving release of the contents of the CTL's granules. The two steps result in the third step which is the disintegration and lysis of the target cells.

This particular mechanism of cytocide depends on the granules present in the CTL's and their contents (119). The granule mediators include perforin, granzymes, TIA-1, and leukalexin. The following is a brief discussion of the more important of these mediators : perforin and granzymes, which are instrumental to the lethal hit delivery.

i. Perforin :

This cytotoxic mediator owes its name to the fact that it can *perforate* target membranes by forming transmembrane pores. Consequently, the targets cells lose their membrane integrity and die of osmotic lysis. Perforin (119)(120) is released from the CTL's granules as monomers. The monomers, in the presence of $Ca^{2+}$, are thought to undergo conformational change and insert in the target lipid membrane. The monomers then aggregate and polymerize to form a central opening (pore), which grows in diameter through the progressive recruitment of additional monomers (5-20 nm). The process is $Ca^{2+}$- and temperature-dependent.

Pores formed by perforin are similar to those formed by the membrane attack complex (MAC) complement components C5b, C6, C7, C8, and C9. In fact, perforin and C9 were found to be structurally and functionally similar. They cross-react antigenically and are

inhibited by high salt concentration. Perforin has been purified from killer lymphocytes of mice, rats, and humans. The mouse and human perforin gene have been both cloned and mapped to chromosome 10.

Perforin appears to be necessary for the function of lymphokine-activated killer cells like NK and $CD8^+$ T-cells, since LAK cells generated from perforin knockout mice were found to have significantly reduced cytotoxicity against tumor target cell lines (121). However, a population of $CD4^+$ T-cells that utilized a perforin-dependent mechanism was also discovered (122).

Interestingly, the role of perforin-dependent cytotoxicity in IDDM was tested in a disease model involving transgenic mice expressing glycoprotein of lymphocytic choriomeningitis virus (LMCV) in the β-cells of the endocrine pancreas (123). Infection of such mice with LCMV leads to diabetes. However, when these mice were crossed with perforin-knockout mice, the resultant mice developed insulitis only but no diabetes upon infection with LCMV. Neither thymic tolerance induction by negative selection nor T-cell repertoire was affected in the perforin-deficient mice. Therefore, perforin-dependent cytotoxicity is not required for the initiation of insulitis but is essential for the destruction of the β-cells. Importantly, perforin GKO mice, backcrossed to NOD mice, are protected from diabetes development (124).

ii. Granzymes :

The delivery of the lethal hit to the target cell involves discharging the contents of the CTL granules. Perforin is thought to create pores in the membrane of the target cell for osmotic lysis and also to facilitate the transfer of other granule CTL contents that induce cell death. Such contents are granzymes (125), which are a family of at least eight serine esterases (granzymes A-H). They comprise approximately 90% of the total granule proteins. The most abundant and most studied of these granzymes are A and B; the homologues of which were also found in humans while the search for the others has yet to yield convincing results.

Granzyme B is an abundant granzyme in CTL granules. It is reported (230) that granzyme B cleaves substrates containing acidic side chains, a feature unique among eukaryotic serine proteinases. The preference of granzyme B to cleave proteins to the carboxyl side of the acidic amino acids is determined by the positive charge in the specificity pocket. Granzyme A, on the other hand, cleaves adjacent to basic residues.

The use of serine protease metabolic inhibitors has indicated the involvement of granzymes in cytolysis (126) (127). More recently, it has been shown that granzyme B could induce DNA fragmentation, indicative of apoptosis, but only when target cells were simultaneously exposed to sublytic quantities of perforin (128). Therefore, it can be theorized that granzyme B is instrumental in the

induction of apoptosis in the target cell and perforin maybe necessary for providing an access for granzyme B into the target cell. However, the role of perforin to provide access for granzyme B into the target cell has been recently challenged (129) since granzyme B was found to be bound and internalized by Jurkat cells independent of perforin presence. Nevertheless, the internalization of granzyme B did not result in toxic consequences and apoptosis only occurred in the presence of perforin. This indicates that perforin, although not necessary for providing an access for granzyme B into the target cell, is essential for the lysis of the target cell.

Granzyme B itself has been localized in the nucleus of the target cell and was recognized to bind to an 80-kilodalton protein from the nuclear extract (130) (131). The particular target for granzyme B has been identified (132) as CPP32, the precursor of the protease responsible for cleavage of poly(ADP-ribose) polymerase, which is involved in the induction of apoptosis. Granzyme B is also thought to upregulate the kinase activity of $p34^{cdc2}$ (133). The expression of this kinase is normally restricted to $G_2/M$ phase of the cell cycle. The aberrant increase of $p34^{cdc2}$ activity, upon treatment with perforin and granzyme B, appears to lead to nuclear disintegration and eventually cell death.

**2. Type 1 cytokines activate CTL cytotoxicity :**

Th1 T-cell subsets and their type 1 cytokines (IFNγ, and IL-2) activate CTL cytotoxicity. In fact, it was shown (134) that expression of these cytotoxic molecules was markedly reduced when activated killer lymphocytes were induced in the presence of pentoxifylline (PTX), a methylxanthine derivative known to inhibit the production of Th1 cytokines IL-2, TNFα, and IFNγ. It is also interesting to note that PTX had no effect on the expression of Fas ligand. Therefore, the results from this study suggest that type 1 cytokines may selectively activate perforin- and granzyme-B-dependent cytotoxicity.

# VI. Hypothesis

The previous sections of this introduction have dealt with reviewing the important literature concerned with IDDM development and mechanisms of islet β-cell destruction. Collectively, this information can be summarized in the following points:

1. IDDM is an autoimmune disease.
2. Both $CD8^+$ T-cells and $CD4^+$ T-cells are required for the initiation and development of IDDM.
3. $CD4^+$ T-cells ($T_{helper}$, Th) are "pivotal" to the development or prevention of IDDM, and hence they are the target of immune

modulation strategies to prevent/block the autoimmune response against islet β-cells.

4. Th1 cells and their type 1 cytokines (IFNγ, IL-2, TNFβ), appear to lead to islet-β-cell destruction, and Th2 cells, with their type 2 cytokines (IL-4 and IL-10) may protect against β-cell destruction.

Based on these points, the following hypothesis is currently the subject of investigation by many laboratories studying the pathogenesis of IDDM :

1. The initial event in IDDM, which is yet to be defined, results in an immunogenic signal which leads to an immune response in the islets. This signal appears to involve the presentation of an islet-β-cell antigen (e.g. autoantigen) on an MHC class II peptide of an antigen presenting cell (APC), to $CD4^+$ T-cells.

2. Also, the APC secretes IL-12 and induces the proliferation and differentiation of the $CD4^+$ helper T-cells into a Th1 subset.

3. The Th1 subset secretes IFNγ and IL-2 to further augment Th1 cell function and activate cytotoxic cells (e.g. $CD8^+$ T-cells and NK) and cytotoxic macrophages. Moreover, the production of IFNγ by Th1 T-cells also leads to the inhibition of the Th2 cells, since these subsets are mutually inhibitory via their respective cytokine products, IFNγ and IL-4.

4. The production of the Th1 response can lead to the destruction of the insulin-producing cells, the pancreatic islet β-cells in one or more of three possible ways :

a. Proinflammatory cytokines (IFNγ, TNFα, and IL-1β in combination) can mediate direct destruction of β-cells.

b. Type 1 cytokines (e.g. IFNγ) can activate the cytotoxic macrophages to produce free radicals ($O_2^{\cdot -}$, $H_2O_2^{\cdot}$, and $NO^{\cdot}$) which can destroy the free radical-sensitive β-cells.

c. Type 1 cytokines (IFNγ and IL-2) can activate the cytotoxic $CD8^+$ T-cells and NK cells to produce cytotoxic molecules (e.g. perforin and granzymes) to destroy the β-cells.

5. The above scenario occurs only in the diabetes-prone animals. However, we hypothesized that immunomodulatory interventions (e.g. administration of Complete Freund's adjuvant, CFA, or theophylline) offer protection against the development of IDDM through the downregulation of Th1 cell function and/or the upregulation of Th2 cell function sufficiently to inhibit the Th1 response and spare the targeted β-cells. The up-regulation of Th2 cell function should be reflected by the secretion of the type 2 cytokines (e.g. IL-4 and IL-10).

Also, since cytokines may be secreted by both $CD4^+$ and $CD8^+$ T-cells, selective deletion of $CD4^+$ T-cells and $CD8^+$ T-cells from DP-BB rats *in vivo* and analysis of cytokine changes in islets and spleens in relation to prevention of diabetes should reveal the relative contribution of $CD4^+$ and $CD8^+$ T-cells in the development of

autoimmune diabetes and the possible cross-regulation of these T-cell subsets.

***For the purpose of this thesis, I chose to concentrate on the pivotal role played by the Th subsets in the development and/or prevention of IDDM through studying their cytokine production patterns during the development IDDM in autoimmune diabetes-prone and diabetes-resistant animals, and diabetes-prone animals manipulated by immuno-modulatory procedures. In addition, I was interested in exploring the secretory granule (perforin/granzyme B) mechanism of cytotoxicity as a terminal step in the pathway for β-cell destruction. The animal model chosen for this endeavor was the Bio-Breeding (BB rat), which is discussed in detail in the next section.***

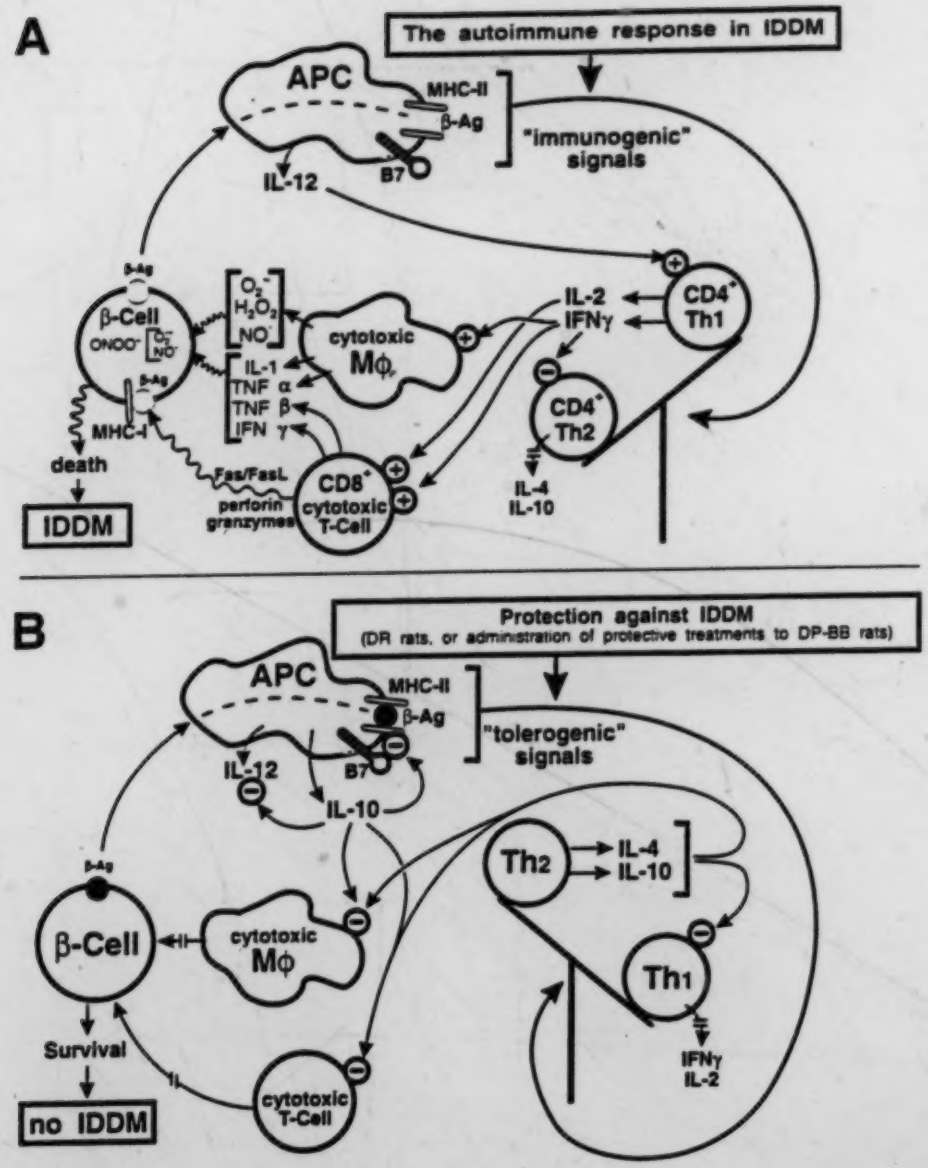



**Fig. 1-1:** Schematic diagrams showing the hypothesized immune response with its cytokine and effector mediators during (A) the development of IDDM, and (B) in the diabetes-resistant (DR) BB rats or during protective interventions in the diabetes-prone (DP) rats. APC = antigen presenting cell, Ag = antigen, B7 = costimulatory molecule B7, (+) = stimulatory signal, (-) = inhibitory signal.

# VII. The BB rat as a model for studying IDDM

## 1. History and discovery :

In 1974, the Chappel brothers discovered spontaneous hyperglycemia and ketoacidosis in a colony of outbred Wistar rats at the Bio-Breeding Laboratories in Ottawa, Canada (135). The animals were found to be hyperglycemic (252-732 mg/dl), hypoinsulinemic (0.1 ng/ml), and hyperketonemic (6-13 mM). These rats were inbred to eventually give what is now known as the diabetes-prone (DP) rat strain. Spontaneous diabetes in DP-BB rats occurs between approximately 50 and 100 days of age, when DP-BB rats from both sexes develop pancreatic insulitis which is followed by selective destruction of the insulin-producing β-cells. The other phenotype, the diabetes-resistant (DR) rats, was developed early in the program of inbreeding by selecting those which failed to developed diabetes at the sixth generation and starting a control line of nondiabetic BB rats. This DR-BB strain has a cumulative incidence of spontaneous diabetes of less than 1%.

The incidence of diabetes in the DP-BB rat depends on the haplotype and the conditions of the colonies where they are raised. Various colonies of DP-BB rats have been established throughout the world (136). Each of these colonies was inbred separately and as a result they have developed into seven unique haplotypes with

different genetic polymorphisms. The rats used in this thesis were directly obtained from the University of Massachusetts Medical centre in Worcester which houses the National Institutes of Health (NIH) breeding colony. The BB rat strains in this colony were derived from a specific program of brother-sister mating and kept under viral-antigen-free (VAF) and specific-pathogen-free (SPF) conditions, where sterilized cages and bedding, cage hoods, autoclaved food (Purina 5010) and acidified drinking water (or tetracycline water) are employed in the care of animals. In addition, gowns, gloves, booties, and masks are used by the caregivers. This is because it was found that the environmental factors (i.e. housing conditions) affected the incidence and onset of diabetes in those animals. Under these conditions, the incidence of diabetes reaches about 90% in the DP-BB rats by the age of 120 days.

Similar to humans, the DP-BB rat develops an abrupt onset of diabetes. The disease onset in the DP-BB rat occurs around the age of approximately 50-100 days, which is characterized by hypoinsulinemia and consequently hyperglycemia. This is accompanied by weight loss, polyuria, polydipsia, glucosuria, and ketosis. The animals usually die within 4-7 days in the absence of provision of exogenous insulin.

## 2. Genetic susceptibility :

The mode of inheritance of diabetes in the DP-BB rat appears to be an autosomal recessive trait with incomplete penetrance (137). As in humans, the most notable genetic markers correlative with IDDM, or autoimmune disorder, are the MHC genes. In the rat, this group of genes are referred to as the RT1 complex. They also consist of the class I and class II antigens. Class I antigens are known as RT1A, RT1C, and RT1E, while class II are RT1B and RT1D which are equivalent to the mouse class II, I-A and I-E, respectively (138).

The RT1 complex of the BB rat is *u* haplotype ($RT1^{u/u}$). Expression of diabetes is independent of class I haplotype, but requires the presence of class II $RT1^{u}$ allele. Breeding studies showed that all the diabetic offspring in the $F_2$ generation resulting from breeding the BB rat with Lewis rat ($RT1^{l/l}$) or with Buffalo rat ($RT1^{b/b}$) carried at least one *u* haplotype (139).

There are also three non-MHC genes that were found to be associated with IDDM. The first and most important is a lymphopenia gene, *lyp*, which results in a severe lymphopenia in the DP-BB. This gene lies on chromosome 4 and is tightly linked to the neuropeptide Y gene (140). Breeding studies (137) showed that the lymphopenia locus always segregated with diabetes and it was the major genetic difference seen between the DP-BB and DR-BB rat phenotypes. It was found to control the intrathymic cytokine ratio in the BB rats (141). The frequency of IFNγ and IL-10 mRNA expressing

cells in isolated thymocytes determined by quantitative image analysis, demonstrated an increased IFNγ/IL-10 ratio in thymocytes from *lyp/lyp* homozygotes compared to *lyp*/+ or +/+. This confirmed a *lyp* gene dose-dependent segregation of the IFNγ$^{high}$ phenotype. The *lyp* gene was inherited independently from the MHC. Besides the *lyp* gene, other studies found two other genes that associated with diabetes : a gene linked to the MHC on chromosome 20 (RT1-B), and a third unmapped gene (140).

### 3. Immunopathology of the BB rat :

#### a. T-cell deficiency :

The BB rat exhibits many immune anomalies that are thought to predispose the animal to the development of diabetes. The first and most important in the development of diabetes is the severe lymphopenia resulting from the expression of the *lyp* gene. The lymphopenia of DP-BB is generally characterized by a substantial reduction in $T_{helper/inducer}$ cells and a nearly complete absence of $T_{cytotoxic/suppressor}$ cells. This is accompanied by an increase in or over-representation of natural killer (NK) cells (142).

It has been reported (143) that the DP-BB rat was profoundly T-cell lymphopenic compared to its nondiabetic counterpart, the DR-BB rat. Using a rat monoclonal antibody that recognized all T-cells, the authors were able to show that the DP-BB rat has only one-third of

the normal amount of lymphocytes found in the control non-diabetic Wistar strain rat. Upon using a $T_{helper/inducer}$-cell marker (W3/25), the authors reported that this T-cell subset comprised only 8% of the total T-cells, whereas 84% of the control rat T-cells were positive for W3/25. Therefore, it was concluded that the DP-BB rat was severely deficient in the production of T-cells in the circulation, specifically $T_{helper/inducer}$-cells. Furthermore, several studies in the literature reported that $T_{cytotoxic/suppressor}$ cell subset ($TCR^+/OX8^+$) was nonfunctional and nearly absent in the DP-BB rat (144), (145), (146). It's also important to note that the lymphopenia observed in the DP-BB rat is specific to T-cells and not to B-cells.

**b. The RT-6 system:**

The T-cell deficiency in the DP-BB rat was found to correlate with the absence of RT-6 T-cell alloantigenic system (147). The RT-6 differentiation alloantigen is a nonglycosylated surface membrane antigen that has a molecular weight of 21 kD. The RT6 gene lies on chromosome 1 in the rat. It is expressed on 60-70% of peripheral T-cells, but is usually absent the surface of bone marrow and thymocytes. Therefore, it's considered a maturation antigen in T-cells. Both T-cell subsets, $T_{helper/inducer}$ and $T_{cytotoxic/suppressor}$ cells, express RT6 after the development of their distinctive W3/25 (i.e. CD4) and OX8 (i.e. CD8) surface markers. The functions of $RT6^+$ T-cells include the participation of graft-versus-host response and

proliferation in a mixed lymphocyte reaction (MLR). Furthermore, the $RT6^+/OX8^+$ T-cells may play a potent suppressor activity in MLR.

The RT6 system (148) has two known alleles, $RT6^a$ and $RT6^b$ which encode RT6.1 and RT6.2 alloantigens, respectively. It appears that while RT6.1 is expressed on the surface of the DR-BB and the Lewis rat's peripheral T-cells, the Wistar-Furth rat expresses the RT6.2 alloantigen. The absence of the RT6.1 in the DP-BB rat is not due to a defect in the structural gene, or transcription and translation of the gene (149). Furthermore, the resultant protein was found to be correctly processed and folded in the DP-BB lymph node cells. Instead, it was found that the RT6.1 protein expressed in the lymph node cells of the DP-BB rat is less than 10% of that expressed in the lymph node cells of the DR-BB rat. The reason for the absence of the RT6 alloantigen from the periphery is probably due to premature death of the T-cell lineage in combination with other factors.

Recent studies (150) indicate that the DP-BB rat contains reduced numbers of T lymphocytes in the periphery. The majority of these T-lymphocytes are recent-thymic migrants (RTM) or immature T-cells. The lack of maturity is defined by the absence of the RT6 and CD45RC differentiation markers and the presence of Thy-1, which is a surface glycoprotein expressed by T-cells that have recently emigrated from the thymus. Compared with the DR-BB rat, the DP-BB rat contains markedly decreased numbers of $Thy1^-$ $RT6^+$ T-cells, especially $CD8^+$ T-cells, and an approximately 3-fold increase in the percentage of $Thy1^+$ $RT6^-$ T-cells, which serve as precursors of both

Thy1$^-$ RT6$^-$ T-cells and Thy1$^-$ RT6$^+$ T-cells. So it appears that besides defective thymic export, most of the immediate descendants of RTMs in the DP-BB rat undergo non-productive proliferation and death 3-7 days post-thymic at which time their counterparts in the DR-BB rat differentiate into Thy1$^-$ RT6$^+$ T-cells. The reason for the deficiency and immaturity of the peripheral T-cells in the T-cells in the DP-BB rat was linked to its failure to produce the distal type II promoter transcript for the p56$^{Lck}$ signal transduction protein kinase (151) which is thought to play an important role in thymocyte ontogeny and activation of mature T-cells (152).

Absence of the RT6$^+$ T-cells and the concomitant lymphopenia may contribute directly to the development of diabetes in the DP-BB rat. When RT6$^+$ T-cells were depleted from DR-BB rats by anti-RT6.1 monoclonal antibody treatment (153), the result was the development of insulitis after 10 days. At this initial stage, the insulitis contained some macrophages but then progressed to be rich in macrophages and T-cells by 18 days after the injection. Therefore, the absence of the RT6$^+$ T-cells in the DP-BB rat appears to be tightly linked with the absence of regulatory T-cells which play an important role in the prevention of the process of insulitis and IDDM as observed in the DR-BB rat.

**c. The presence of autoantibodies in the BB rat :**

The presence of autoantibodies directed against the surface of the islet cells of the DP-BB rat was documented in two studies. The first study (154) examined the presence of **ICSAs** and spleen lymphocyte autoantibodies in the sera of prediabetic and acutely diabetic BB rats as well as non-BB Wistar rats. The autoantibodies to islet and spleen were found in sera of the diabetic rats when tested against the islet and spleen dispersed cells at the rate of 85% and 93%, respectively. In addition, there was strong positive correlation between the two types of autoantibodies in the diabetic animals. Both the non-diabetic BB rats and the non-BB rats were negative for both types of autoantibodies.

The second study (155) utilized immunofluorescence technology to detect the binding of autoantibodies against the individual islet cells. The study confirmed the findings of the previous one by examining the presence of the ICSAs in the sera of prediabetic animals. The autoantibodies of interest were found in the sera of predisposed animals up to 6 weeks before the onset of disease. Only one control (Wistar) rat was found positive for the ICSAs compared with all DP-BB rats tested.

In addition to the ICSAs, autoantibodies against the intracellular components of the islet cells are found in the BB rat. Similar to what was found in humans, autoantibodies to GAD proteins were present in the sera of DP-BB rats even before the onset of

diabetes (29) but not in the control (Wistar) rats. Furthermore, the autoantibodies were reported to be against two forms of the islet protein, $GAD_{65}$ and $GAD_{67}$ (157).

Other studies reported that autoantibodies against smooth muscle and thyroid colloid were present with increased frequencies in the diabetic BB rats (158). This is not surprising since the presence of autoantibodies against several organs is commonly observed in autoimmune disorders.

**d. Insulitis:**

Similar to what is observed in humans and the NOD mouse, the presence of inflammation of the pancreatic islets was also seen in the BB rat. The insulitis in the DP-BB rat starts around the age of 40 days and is composed of monocytes/macrophages, $CD8^+$ and $CD4^+$ T-lymphocytes. However, studies (160) utilizing a monoclonal antibody specific for macrophages (MRC OX41) in the BB rat suggest that monocytes/macrophages were the first infiltrating cells in the lesion. Mixed immunocytes including macrophages, T-, B- and NK cells followed at later stages. It appears that the professional antigen-presenting dendritic cells were the first to accumulate around the islets while scavenger macrophages and MHC II-positive β-cells were only seen late in the disease (161). The other major component of the insulitis lesion during prediabetes is NK cells; however, NK cells have been excluded as mediators of β-cell destruction in DP-BB rats (53).

Different populations of T cells were reported (162) during the onset of diabetes. Analyses of T-cell subsets revealed that $CD4^+$ T-cells were the predominant subset (50-55%), followed by the double negative ($CD4^-$ $CD8^-$ T-cells) T-cells (25-30%) and $CD8^+$ T-cells were also present in significant quantities (15-20%).

Minimal peri-insulitis is also found in the DR-BB rat; however, this does not progress to IDDM, probably due to the interference of the regulatory $RT6^+$ cells, hence sparing the β-cells.

**4. IDDM as T-cell mediated disease in the BB rat :**

The aforementioned immunopathological signs are only indirect evidence for the basis of autoimmunity of IDDM in the DP-BB rat. The direct evidence usually stems from the central role played by the T-cells in the development of the disease. The first piece of evidence was provided when complete or partial thymectomy was performed on BB rats (34). The surgery was performed within 24 hours of birth and resulted in reduction in the incidence of diabetes in the animals with complete thymectomy by 9 fold compared with the control animals that received sham surgery. Inadvertent partial thymectomy also provided significant protection (3 fold). Therefore, the thymectomy removed the source of the autoreactive T-cells which result in IDDM in the DP-BB rat.

It was also found that passive transfer of Concanavalin A-treated spleen cells from diabetic BB animals into young DP-BB rats resulted in the development of severe insulitis and diabetes (37). However, the study also showed that when other recipients, including BB rats with low-incidence of diabetes, were used, diabetes did not develop. This indicates that the diabetic BB rat Concanavalin-A-treated spleen cells possess the ability to initiate the development of disease but are not sufficient for the massive destruction of β-cells and the diabetes that ensues.

In addition, administration of antiserum to lymphocytes alone for one month prior to or at the time of onset of the disease normalized the hyperglycemia in 36% of already diabetic animals and prevented the development of permanent diabetes in susceptible prediabetic ones (44). Other studies which involved the administration of monoclonal antibodies directed against key T-cell subsets to DP-BB rats have also resulted in the prevention of IDDM (163) (53) (60). This area will be discussed in further detail in chapters 5 and 6. Similar results were also observed with bone-marrow stem cell transplantation (164) and the administration of the immunosuppressive drug CsA (43).

The role of macrophages and their cytokines in the development of Kilham rat virus (KRV)- induced diabetes in the DR-BB rat was also investigated (85). KRV infection caused diabetes in the DR-BB rat and macrophages-derived cytokines (IL-12, TNFα, and IL-1β) measured in splenic lymphocytes and pancreatic islets were

elevated. Depletion of KRV- and poly (I:C)-treated DR-rat macrophages with liposome-encapsulated dichloromethylene diphosphonate (lip-$Cl_2$MDP) resulted in complete prevention of insulitis and diabetes in the treated DR-BB rats. Furthermore, depletion of macrophages before the isolation of lymphocytes from DR-BB rats treated with KRV and Poly (I:C) resulted in the loss of ability to transfer diabetes to young DP-BB rats. These studies suggest an important role of macrophages in initiation of diabetes as well as diabetes transfer.

T-cells products, cytokines and cytotoxic molecules, were also found in the insulitis lesion in the BB rats (47)(48)(50). However, the literature in this area is limited compared with the reports in the NOD mouse.

***This thesis will deal mainly with the role of T-cells and their cytokine and cytotoxic products in the pathogenesis of IDDM in the BB rat. I will describe my investigations into the aforementioned hypothesis of the pivotal role of the Th T-cell subsets, and their type 1 cytokines, in the development and/or prevention of IDDM in the autoimmune diabetes-prone BB rat.***

# Chapter 2

# Materials and Methods

# I. Animals and tissues used for studies

## 1. Animals, diet, and housing conditions :

Diabetes-prone (DP-) and diabetes-resistant (DR-) BB/Wor rats were purchased from the National Institutes of Health contract colony at the University of Massachusetts Medical school, Worcester, Massachusetts. The rats were housed in the viral-antibody free (VAF) animal facility in the Heritage Medical Research Centre, University of Alberta, under specific pathogen-free conditions throughout the studies. The animals were cared for according to the guidelines of the Canadian Council on Animal Care, in which sterilized cages and bedding, cage hoods, autoclaved food (Purina 5010) and acid drinking water were employed in the care of animals. In addition, gowns, gloves, booties, and masks were used by the caregivers.

The animals were given the various treatments (see below), and monitored daily from age 50 days for the development of diabetes by urine and blood glucose tests (see below) as well as monitoring loss of weight gain. The rats were euthanized at the age of 60-75 days by sodium pentobarbital overdose (30 mg of Somnotol /kg of body weight ).

**2. Organization of rat groups in studies and tissue harvest :**

For each study, three groups of rats were used. The first group of the rats was followed for diabetes incidence from 50-140 days of age. The second group were sacrificed between the ages of 60 to 80 days and their tissues were used for histological analysis and determination of insulin content. The third group were also sacrificed at the same age and their tissues were used for gene transcription or cellular subset analysis depending on the study.

**a. Testing for the diabetes onset in BB/Wor rats :**

Diabetes incidence was followed in the first group of rats (number of animals (n) depends on study), through monitoring of three parameters :

**i. Body Weight :**

Beginning when DP-BB rats were 50 days old, they were weighed three times weekly. Weight loss, or loss of weight gain as the rats are still growing at 50 days of age, is an indication of impending IDDM (This procedure was done by Wilma Suarez-Pinzon).

**ii. Glucosuria testing :**

The urine samples were obtained between 7:00-10:00 a.m. which should reflect the glycemic state of the animal during the previous night's feeding state since rats are nocturnal feeders. Urine was expressed from the bladder manually by gentle pelvic compression against the pubic symphysis, not intestine. Once obtained, the urine glucose level was determined with Tes-Tape (Ely Lilly), if the tape turned green, it was considered to be a positive result for diabetes. As a result of a positive glucosuria test (i.e. green Tes-Tape), the blood glucose value was then determined.

**iii. The measurement of blood glucose level :**

The blood glucose level was monitored using the Accu-chek II glucometer (Bayer). A small portion of the rat tail was punctured with a 22-gauge needle and a drop of blood was obtained. The bleeding was stopped with a silver nitrate applicator. Using code 880 glucometer strips, the blood glucose level (BGL) was obtained. If found to be more than 12 mmol/L for two consecutive days, accompanied by polyuria and weight loss as well as glucosuria, the rats were considered diabetic (This procedure was done by Wilma Suarez-Pinzon).

**b. Rats used for histological analysis and determination of insulin content :**

A small (10%) part of the pancreases of the rats in the experimental or control group was harvested for histological examination after hematoxylin and eosin (H&E) stain or for insulin staining according to the following procedure. The pancreatic tissue was harvested and fixed in 10% formalin, embedded in paraffin, sectioned at 4.5 μm and stained with anti-insulin antibody (Dako, Carpinteria, CA) using an immunoperoxidase technique. Coded slides were read by light microscopy. The rest of each pancreas (90%) was weighed, minced with fine scissors, sonicated and incubated at 4°C for 24 hr in acidified ethanol (75% ethanol, 1.5% 12 mol/HCl, and 23.5% $H_2O$) to extract insulin and to determine the pancreatic insulin content by radioimmunoassay using a kit for rat insulin (Pharmacia, Uppsala, Sweden) (This procedure was done by Wilma Suarez-Pinzon).

**c. Rats used for Flow Cytometry Analysis (FACS) and PCR analysis :**

The third group of rats were sacrificed and the tissues were harvested for either mononuclear leukocyte subset analysis by FACS, or gene transcription (mRNA) analysis by PCR. Both procedures are described in detail in the next sections.

# II. Treatments administered

We have decided to investigate the effect of immunotherapeutic interventions on the development of IDDM, pancreatic histology (insulitis), mononuclear leukocyte subsets, and the type 1 / type 2 cytokine profiles in pancreatic islets and spleens in the DP-BB rat. The following is a brief description of the protocol involved in each treatment:

## 1. Complete Freund's Adjuvant (CFA) :

DP-BB rats were injected intraperitoneally once with 100 µl CFA (Difco) /50 g of body weight at the age of 25 days to prevent the development of diabetes as reported before (165). The DP-BB rat control group were injected with phosphate-buffered saline (PBS). Please refer to Fig. 3-1 for experimental protocol.

## 2. Theophylline :

Theophylline (Sigma Chemicals Inc.) was administered to DP-BB rats in their drinking water at 2 mg/ml during a full-day period from 25 until 130 days of age. The drinking water was refilled daily to maintain the stability of theophylline. The levels of theophylline in the rats were measured by HPLC testing of the blood of experimental

animals (166). The control group was given plain water. Please refer to Fig. 4-1 for experimental protocol.

### 3. Monoclonal antibodies to T-cell subsets:

DP-BB rats were injected intraperitoneally with hybridoma cell line supernatants (refer to Table 2-1). The supernatants were prepared as described below, and 2 ml of mouse B-cell hybridoma supernatant containing monoclonal antibodies (mAbs) to rat T-cell subsets (OX-21 control hybridoma, OX-8 (mAb to $CD8^+$ cells) and OX-34 (mAb to $CD2^+$ cells) ) were injected intraperitoneally daily from 25 to 130 days of age (refer to Fig. 5-1 for experimental protocol).

The hybridoma cell lines were obtained originally from the laboratories of Dr. W. Mason, Oxford University, and provided to our laboratory by Dr. A.A. Like, University of Massachusetts Medical School. The hybridoma cell lines (Table 2-1) were frozen and kept in liquid nitrogen (-87°C) in 1 ml aliquots (5-10 x $10^6$ cells). After thawing, 1 x $10^6$ cells were grown in 40 mls of RPMI 1640 medium containing10 mM HEPES, and supplemented for the first week with 50% FBS. The cells were cultured in T150 $cm^2$ flasks maintained at 37°C and 5% $CO_2$/95% air in humidity chamber incubators. The cultures were fed three times a week with the same medium until viability was observed to be approximately 90%. The amount of FBS was reduced gradually for each week in the following order 40%,

20%, 10%, 5%, and finally 1% (the lowest percentage of serum necessary to maintain the production of antibody from the cells). The cells were then transferred to a round sterile spinner flasks (with a magnetic stirrer to stir the culture gently during growth and ensure mixing of growth factors in the medium) at a concentration of $10^7$ cells/ml in 300 mls of media (i.e. total of 3 x $10^9$ cells) and grown for no more than 1-3 days for maximum growth and antibody production (i.e. growth plateau and antibody saturation). By this point the medium color was yellow and culture was turbid. Cell viability was 60-70% and the antibody supernatant was ready to be harvested. The supernatants were spun down at 10,100g at 4°C for 2 hours in the high-speed Beckman centrifuge to remove cell debris and the screening assays (Appendix 3) were performed to select hybridoma supernatant containing high antibody concentration. 40-60 litres were needed for each monoclonal antibody study (OX8, OX21, OX34). These were stored in 30 ml aliquots at -86°C until ready to use. 1 ml from each batch was frozen separately for screening assays in order to test the quality of the supernatants and determine the concentrations of the total proteins and the specific IgG antibody of interest (for results and calculations see Appendix 3).

The amount of the total protein was measured using the Micro BCA Protein Assay Kit (Pierce) in each hybridoma supernatant and determined to be 1,040 µg/ml, 1,180 µg/ml, and 1,590 µg/ml for OX8, OX21 and OX34 hybridoma supernatants, respectively. The amounts of IgG1 antibody in OX8 and OX21 hybridoma supernatants were determined by ELISA to be 59.8 µg/ml and 80 µg/ml,

respectively. Similarly, the amount of IgG2a antibody in OX34 hybridoma supernatant was determined by ELISA to be 106.5 µg/ml. These amounts represent 5.77%, 6.78 %, and 6.91 % of the amounts of total protein injected daily (This procedure was done by Wilma Suarez-Pinzon, Amina Gowa, and Mark Labbe).

## Table 2-1

The monoclonal antibodies used in antibody injection studies and in the FACS analysis of the islet and splenic mononuclear subsets

| Rat Ab | Isotype | Specificity | Reference |
|---|---|---|---|
| FITC | FACS | buffer with Ab isotype control (Rat IgG1 or 2a) | |
| OX1 | IgG1 | CD45 (leukocyte common antigen) | (167) |
| OX8 | IgG1 | CD8 (Tc/s, NK, thymocytes) | (168) |
| OX12 | IgG2a | mouse anti-rat kappa chain (B-cells) | (169) |
| OX19 | IgG1 | CD5 (peripheral T-cells and thymocytes) | (170) |
| OX21 | IgG1 | anti-human C3b inactivator | (171) |
| OX34 | IgG2a | CD2(LFA2) (T, NK, thymocytes, sp. macrophages) | (172) |
| OX42 | IgG2a | brain macrophage and endothelium | (173) |
| 3.2.3 | IgG1 | NK cells | (54) |
| W3/25 | IgG1 | CD4 (T-helper, peritoneal macrophages) | (174) |

# III. Islet mononuclear leukocyte isolation and preparation for subset analysis or gene studies

## 1. Isolation of pancreatic islets :

Islets were isolated from the pancreas by addition of collagenase (from Sigma, at a concentration of 1 µg/mg of pancreas) to small cut pieces of the pancreas in a glass beaker on ice. Individual rat pancreases were prepared separately. The digestion was carried out by addition of 15 ml of cold serum-free RPMI medium, then incubation into the 37°C water bath for 5 minutes while shaking, and the beaker was maintained moving inside the water bath. The digestion was stopped by the addition of 25 ml of RPMI (10% FCS) at 4°C. The digestion was transferred to a 50 ml conical tube and spun at 400g in a clinical centrifuge for 5 minutes at room temperature. After washing 3 times with 25 mls RPMI (10% FCS) and finally resuspending in 5 ml of RPMI (10% FCS), 10 mls of Ficoll (25% of HISTOPAQUE-1077, from Sigma Diagnostics) was added on top of the resuspended pellet followed by 10 mls of RPMI (10% FCS). The homogenate was spun down at 400g in a clinical centrifuge for 5 minutes at room temperature and the islet ring, containing the islets and the infiltrating leukocytes, was harvested. After washing 3 times with 10 ml of RPMI (10% FCS), the islets were hand-picked under a stereoscopic microscope, and the total number of islets per pancreas was recorded.

**2. Isolation of islet-infiltrating mononuclear leukocytes (MNLs):**

To isolate the infiltrating MNLs from the islets, the islets from individual pancreases were kept separate and dissociated by addition of 2 ml of enzyme-free Hanks' base dissociation buffer containing 3 mM EGTA, pH 7.4 for 30 minutes at 37°C in a glass beaker in a shaking water bath. After stopping the reaction by adding 15 mls RPMI (10% FCS) at 4°C, the homogenate was transferred to a 50 ml tube and washed twice with the 15 mls RPMI (10% FCS) at 4°C and spun down each time at 400g for 10 minutes. The supernatant was decanted and the pellet was transferred to a serum-coated 13x100 round-bottom polypropylene tube with 6 mls of RPMI 1640 (10% FCS). The islet preparation was mechanically disrupted by passing through needles gauges 9, 16, and 22 in order. The homogenate was spun down at 400g in a clinical centrifuge for 5 minutes at room temperature. The supernatant was removed and the cells were resuspended in 2 ml of 65% Percoll (Pharmacia LKB Biotechnology) and 2 ml of 55% Percoll, 2 ml of 45% Percoll, and 2 ml of RPMI (10% FCS) were successively layered on top. The gradient was spun at 500g for 20 minutes with no brake in the clinical centrifuge. The desired interface was harvested (leukocytes should be at the bottom and at the interface between 65% and 55% Percoll, and the islet endocrine cells should be at the interface between 55% and 45% Percoll). Each desired fraction was washed twice with 15 ml RPMI (10% FCS). The cell fractions were stained separately with

trypan blue to determine cell viability. On the day of use, 1 part of the cell suspension (2-5x $10^6$ cells /ml) was added to 1 part of the following solution: 4 parts of 0.2% (w/v) of Trypan blue in water were mixed with 1 part of 5x saline. The cells were loaded into a haemocytometer chamber and a total of > 200 cells were counted. Viable cells/ml = (average number of cells in large square) x $10^4$ / ml x 1 dilution, and % viability = (Number of viable or unstained cells/ Total number of cells ) x 100%. The cells were used only if viability exceeded 80%. Two fractions were obtained per islet preparation : 1. islet endocrine cell-enriched fraction, and 2. Mononuclear leukocyte (MNL)-enriched fraction. The MNL samples were analyzed by FACS, Giemsa staining, and PCR.

## IV. Analysis of mononuclear leukocyte subsets

A cytospin was prepared from peripheral blood leukocyte fraction after Ficoll purification. The cells were counted using Giemsa staining to differentiate the leukocyte subtype. The splenic and islet mononuclear cell subsets were analyzed using flow cytometry analysis.

**1. Giemsa Staining:**

Glass slides were coated with 3-Aminopropyltriethoxysilane (Sigma Chemicals), and 50,000 cells were layered onto the slides and attached by cytospin. Using Leukostat Stain Kit from Fisher Scientific, the slides were dipped in the fixative solution for 5 seconds, in Solution I (eosin) for 3 seconds and finally in Solution II (Methylene blue) for 2 seconds. The slides were rinsed in water until almost clear and let air-dry. For each individual rat sample, 2,000 (1,000 x 2 wells) cells were counted (using light microscopy, 100x oil immersion), and leukocyte types (lymphocytes, monocytes, neutrophils, eosinophils, and basophils) were enumerated and recorded as percentages of the total leukocytes.

**2. Flow Cytometry Analysis (FACS) :**

The FACS analysis of the islet and splenic mononuclear cell subsets involved the binding of the primary monoclonal antibody (cell-surface marker specific) to the target cell. This was followed by the binding of the secondary antibody conjugated to FITC.

The cells of interest were added at 0.3 x $10^6$ cells/well to non-coated non-sterile 96-well plates. 100 µl of FACS buffer containing 1-10 µg of primary antibody (mouse anti-rat) or mouse isotype control antibodies (see Table 2-1) were added to each well and incubated at 4°C overnight. The FACS buffer was made up of PBS, pH

7.4, 0.02% $NaN_3$, and 0.05% bovine serum albumin (BSA) which functions as a stabilizer and to block non-specific binding. The plates were spun at 170g for 10 minutes at 4°C in a floor centrifuge (International Equipment Company, PR-7000). The supernatant was aspirated and the pellets were washed three times with working buffer (170 µl/well) using a multi-pipette washer containing 12 tips.

The FITC-conjugated secondary antibody (goat-antimouse) (3 mg/ml) was diluted 1/300 and 100 µl were added to each well (i.e. 1 µg /well), mixed well and incubated one hour in the dark, then washed three times as before. The cell suspension was then fixed using 1% buffered formalin (100 µl/well from 5% formalin in PBS, pH 7.4). The fixed cell suspension was transferred to a 12x75-polystyrene tube and the well was washed with 2x100 µl of 1% buffered formalin and the wash was added to the tube (the final sample volume should be 300 µl). The tubes were capped well and covered with aluminum foil, due to the light-sensitivity of the reagents, and stored at 4°C until ready to be analyzed by FACS. The samples were run through the FACScan Flow Cytometer (Becton Dickinson, Mountain View, California). The sample cell fluorescence was analyzed using a FACScan flow cytometer interfaced to a Hewlett-Packard 310 computer. Data analysis was performed using Lysis II software Logarithmically Amplified Fluorescence. Data were collected on 5,000 cells from the islet leukocyte cell samples, and 10,000 leukocytes from spleens and from peripheral blood.

# V. Levels of steady-state mRNA analysis

## 1. Total RNA extraction:

Total RNA was extracted from cells according to a procedure adapted from Chirgwin etal (1979) (175). Total RNA of interest was extracted by adding the cells to 800 µl of 4M guanidine thiocyanate (ICN Biochemicals Inc.), 10 µl 30% antifoam A (Sigma Chemicals Co.), 5.8 µl 2-β-mercaptoethanol (Sigma Chemicals Co.), and 5 µl of yeast tRNA (10µg/µl). The mixture was homogenized by passing through 18-, 21-, then 25-gauge needles 10 times each. The cell homogenate was precipitated, after adding 0.75 volume of redistilled ethanol (95%) and mixing well, by incubating at -20°C for 2 hours and spinning down at 16,000g in an Eppendorf centrifuge (Model #5402) for 30 minutes at 4°C . The supernatant was decanted and the pellet was re-extracted with 500 µl of 19:1 8M guanidine thiocyanate.HCl: 0.5M EDTA pH 8.0. The pellet was passed through 25-gauge needles 10 times, and 0.5 volume redistilled ethanol (95%) was added. After precipitation as before, the extraction procedure was repeated once more. The final pellet was rinsed with RNase-free 70% ethanol and partially dried under vacuum.

To further purify the RNA from proteins and RNases, the pellet was resuspended in 500 µl of RNase-free water and 500 µl of phenol/chloroform (Fisher Scientific) was added, vortexed well, and spun down for 1 minute at 16,000g at room temperature in an

Eppendorf centrifuge (Model #5415 C). The aqueous phase was transferred to a second 500 µl of phenol/chloroform. The process was repeated and the primary aqueous phase was transferred to 800 µl of chloroform. The primary organic phase was re-extracted with 300 µl of RNase-free water and then the secondary organic phase. The secondary aqueous was added to the final 800 µl of chloroform and the primary aqueous phase. The mixture was vortexed and spun down for 1 minute at 16,000g at room temperature. The final pellet was precipitated by adding 1/10 of the volume 3M sodium acetate pH 4.8 (Fisher Scientific) and 2.5 volumes of RNase-free 95% ethanol, vortexing, and by incubating at -20°C for 2 hours then spinning down at 16,000g in an Eppendorf centrifuge (Model #5402) for 30 minutes at 4°C. The final pellet (total RNA) was rinsed with RNase-free 70% ethanol and partially dried under vacuum. The pellet was resuspended in 20 µl of RNase-free water and the cDNA reverse transcription was carried out promptly.

**2. cDNA reverse transcription of total RNA :**

To reverse transcribe the messenger RNA included in the total RNA sample, 5 µl (500 ng) of oligo-dT (Gibco-BRL) was added to the total RNA sample in a 1.5-ml Eppendorf tube and heated at 65°C for 10 minutes then left to cool down to room temperature for 20 minutes. The heating denatures RNA and the cooling allows the reannealling of RNA's poly-A-tail with the oligo-dT primers.

To this mixture, the following reagents were added in order: 5 µl of RNase-free water, 5 µl of 10 mM DTT (Promega), 10 µl of 5X cDNA synthesis reaction buffer (Gibco-BRL), 1 µl of 25mM deoxynucleotide mixture (Promega), 1 µl of RNasin (Promega), and 2 µl of 1/10 dilution of Superscript reverse transcriptase (Gibco-BRL). The reaction was incubated at 37°C for at least 3 hours, then the reaction was stopped by incubating at 65°C for 10 minutes then the sample was frozen in -70°C until PCR analysis.

### 3. Amplification of mRNA sequence using polymerase-chain reaction (PCR)

The message of interest was amplified using PCR technology (176) shown in Fig. 2-1 as a flow chart. The PCR methodology involved design and synthesis of primers, adjusting the conditions for optimum PCR amplification of cDNA, annealing temperature for each primer pair, running positive control sample with the established PCR conditions, detection of the amplified signal with the help of a radioactive nucleotide, and finally running experimental samples in at least two separate PCR runs, at three different dilutions.

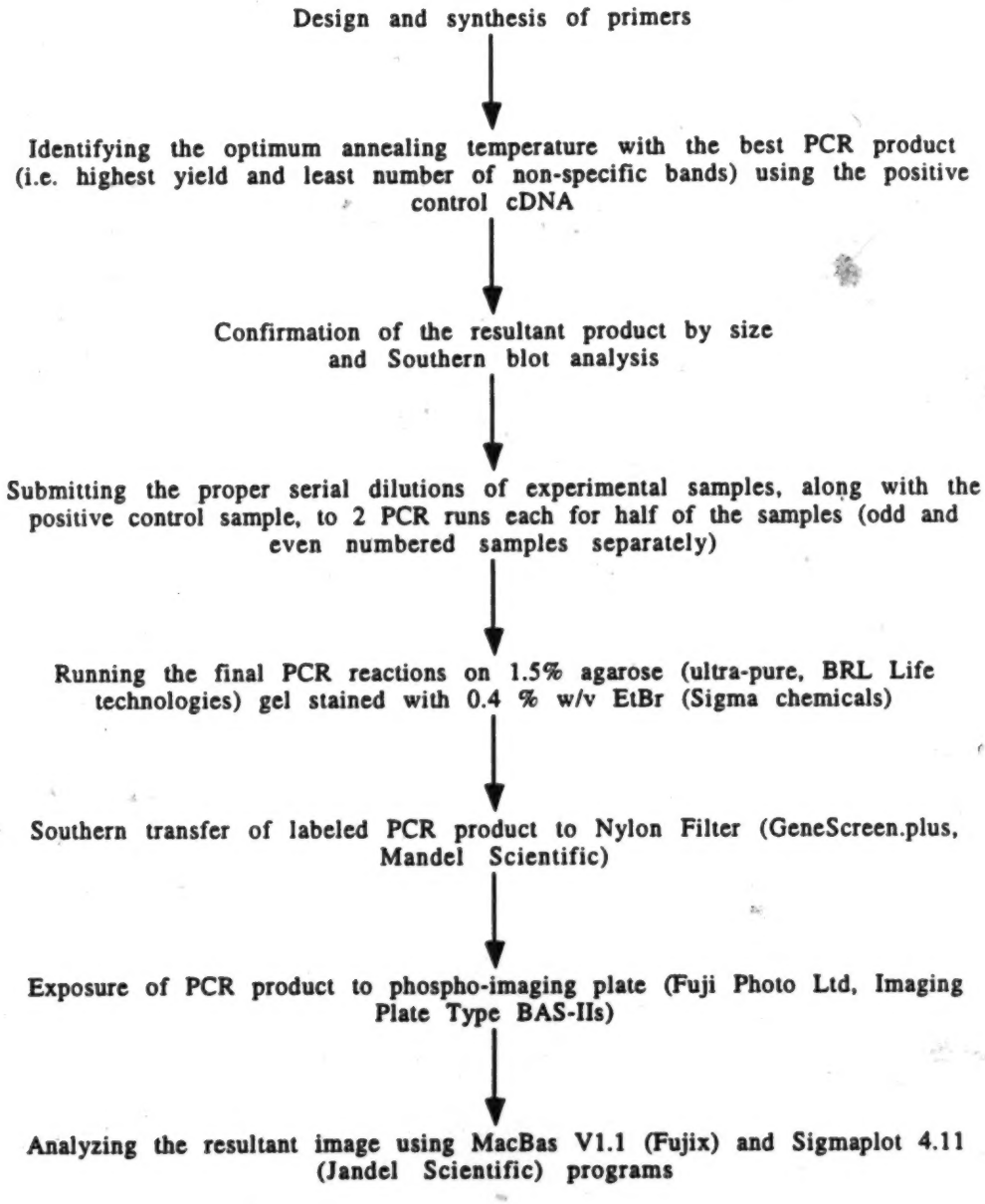

Design and synthesis of primers
Identifying the optimum annealing temperature with the best PCR product (i.e. highest yield and least number of non-specific bands) using the positive control cDNA
Confirmation of the resultant product by size and Southern blot analysis
Submitting the proper serial dilutions of experimental samples, along with the positive control sample, to 2 PCR runs each for half of the samples (odd and even numbered samples separately)
Running the final PCR reactions on 1.5% agarose (ultra-pure, BRL Life technologies) gel stained with 0.4 % w/v EtBr (Sigma chemicals)
Southern transfer of labeled PCR product to Nylon Filter (GeneScreen.plus, Mandel Scientific)
Exposure of PCR product to phospho-imaging plate (Fuji Photo Ltd, Imaging Plate Type BAS-IIs)
Analyzing the resultant image using MacBas V1.1 (Fujix) and Sigmaplot 4.11 (Jandel Scientific) programs

**Fig 2-1 :** Flow chart showing the general steps of the PCR procedure followed in this thesis.

**a. Design and synthesis of primers :**

The sequences of primers for the cytokines, CTL molecules, and house-keeping genes were obtained from commercially available primers, directly from the data banks, or designed based on homology between mouse and rat sequences. Table 2-2 shows the primers used in these studies along with their sequence, the source of the sequence, and the length of the expected fragment. The primers were synthesized in the DNA Synthesis Lab, Department of Biochemistry, University of Alberta. The sequences of the PCR products of interest were confirmed using the expected size of the fragments, size of fragments amplified using internal primers to the original ones, or Southern blot analysis using internal probes.

# Table 2-2

**DNA Sequences for the oligonucleotides used as primers during PCR amplification of the target sequences, and as probes for sequence confirmation by Southern analysis**

| | A | B | C | D | E |
|---|---|---|---|---|---|
| 1 | Target | Primer Sequence | Source | Frag't | Anneal |
| 2 | mRNA | (Southern Probe Sequence) | | Size | Temp |
| 3 | | | | (bp) | (C) |
| 4 | | | | | |
| 5 | Cyp | 5'end GAC AGC AGA AAA CTT TCG TGC | mouse | 276 | 60 |
| 6 | | 3' end TCC AGC CAC TCA GTC TTG G | sequence | | |
| 7 | | (5'TGGTGGCAAGTCCATCTACGGAGAG) | | | |
| 8 | IFNg | 5'-ATC TGG AGG AAC TGG CAA AAG GAC G | rat sequence | 288 | 60 |
| 9 | | 3'-CCT TAG GCT AGA TTC TGG TGA CAG C | Clonetech, Inc | | |
| 10 | | (5'CATCAGCAACAACATAAGTGTCATC) | | | |
| 11 | IL-1b | 5'-GCC ACA GGG ATT TTG TCG TTG CTT | Data bank | 500 | 58 |
| 12 | | 3'-ATG CGA ACT BTC CCT GAA CTC AAC | | | |
| 13 | | (5'GCCAGGAGATGTGTGCTATGTGGCT) | | | |
| 14 | IL-2 | 5'-CAA AGG AAA CAC AGC AGC ACC TGG | rat sequence | 342 | 60 |
| 15 | | 3'-TCC TCA GAA ATT CCA CCA CAG TTG C | Clonetech, Inc | | |
| 16 | | (5'CTCGGAGCTCTGCAGCGTTGTTTGG) | | | |
| 17 | IL-4 | 5'-GTT CTG CTT TCT CAT ATG | Data bank | 330 | 60 |
| 18 | | 3'-AGC GTG GAC TCA TTC ACG | | | |
| 19 | | (5'CAGCCCTCTGAGAGAGATCATCAAC) | | | |
| 20 | IL-10 | 5'-GAG TGA AGA CCA GCA AAG GC | Data bank | 436 | 60 |
| 21 | | 3'-TCA TTC ATG GCC TTG TAG ACA C | | | |
| 22 | | (5'TCAAGTTTTACCTGGTAGAAGTGAT) | | | |
| 23 | IL-12p4 | 5'-AGC TTT TGT GAC AAC CAA TAA GAA | mouse | 300 | 58 |
| 24 | (p40) | 3'-CAA ACT CAC AGA GAT CTG CCT G | sequence | | |
| 25 | | (5'TTAGAAGTGATATTGATAAGAAACC) | | | |
| 26 | TNFa | 5'-TAC TGA ACT TCG GGG TGA TTG GTC C | rat sequence | 295 | 60 |
| 27 | | 3'-CAG CCT TGT CCC TTG AAG AGA ACC | Clonetech, Inc | | |
| 28 | | (5'GAGTGACAAGCCCGTAGCCCACGTC) | | | |
| 29 | TGFb1 | 5'-TGA GTG GCT GTC TTT TGA CG | mouse | 270 | 58 |
| 30 | | 3'-TTG GTA TCC AGG GCT CTC C | sequence | | |
| 31 | | (5'GCCAGGAGATGTGTGCTATGTGGCT) | | | |
| 32 | Perf | 5'-CCT CTT TCC ACC AGA CCT ACC | mouse | 352 | 58 |
| 33 | | 3'-CAG TCC TGG TTG GTG ACC TT | sequence | | |
| 34 | | (5'GGTGGACTACAGCCTGGAGCCCCTG) | | | |
| 35 | Gran | 5'-ATG GTA AAA TGC ATT CCC CA | mouse | 170 | 58 |
| 36 | B | 3'-CAC GTG GAG GTG AAC CAT C | sequence | | |

**b. Adjusting PCR conditions to optimum amplification :**

**i. Buffer, $Mg^{2+}$, dNTP, radioactive dCTP, primers, and enzyme:**

The optimum concentrations of the PCR reagents were determined to be 2 µl of 10X PCR reaction buffer (Perkin-Elmer/Cetus, Norwalk, CT), 60 mM of $MgCl_2$, 0.25 mmol/l of each dNTP (Boehringer Mannheim), 2.5 µCi of [$\alpha$-$^{32}$P]dCTP (3,000 Ci/mol; Dupont-NEN, Mississauga, Ontario), 80 ng of each primer, and 1 unit of AmpliTaq (Perkin-Elmer/Cetus, Norwalk, CT).

**ii. Number of cycles :**

Gradually increasing cycle numbers were used initially to determine the optimum number (Fig 2-2). The number of cycles, 40, was chosen based on the fact that it was the highest number on the exponential part of the curve. In addition, a single copy of double stranded DNA (dsDNA) of 300 bp (approximately the average length of the target sequence to be amplified) going through 40 cycles of replication with an assumed replication efficiency of 80%, yields about 1.6 ng of PCR product. Keeping in mind that a 100 ng band of cold DNA is clearly visible on agarose gel stained with ethidium bromide (EtBr), the labeling and phospho-imaging of the product should, in theory, facilitate the detection of product from a single copy. It was determined in our lab that a single copy (0.001 fg) of

dsDNA of approximately 300 bp used as starting material in a 40-cycle PCR run yields a product that is about 5 ng which was detectable by radioactivity if labeled (~745 cpm). For calculations see Appendix 1.

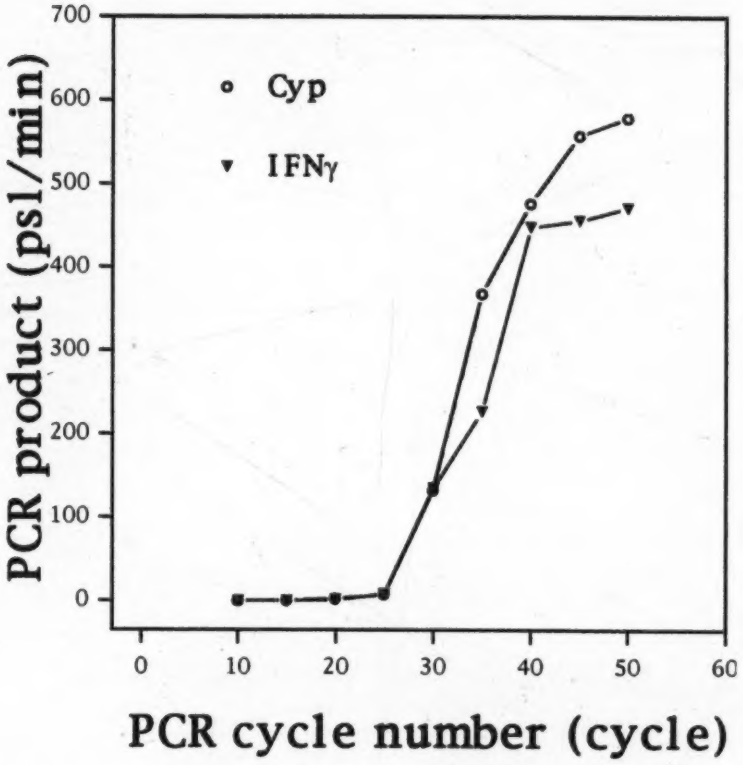



**Fig. 2-2 :** Amplification of cyclophilin (Cyp) and interferon gamma (IFNγ) message from 30 ng of Con-A-activated splenocyte nucleic acids with increasing PCR cycle number. The PCR product is expressed in photo-stimulated luminescence/exposure time (psl/min).

**iii. cDNA concentration:**

The optimum concentration of template used as a starting material for amplification was determined (for Cyp and IFNγ) after submitting serial dilutions of the final reverse transcription reaction mixture to 40 cycles of PCR amplification at 94°C for 20s, 60°C for 20s, and 72°C for 30s, using the Gene Amp System PCR 9600 (Perkin-Elmer/Cetus). The primers used in this test were specific for cyclophilin (Cyp), as a house-keeping gene or internal control, and an example of a cytokine gene, IFNγ. The optimum concentration of template was investigated using serial dilutions of Con-A-activated DR-BB rat splenocyte cDNA. The latter was also used as a positive control sample for all the PCR experiments as the Con-A activation resulted in the upregulation of cytokine gene transcription. The advantage of using such a sample as a positive control is that it represents a tissue sample from the same animal source as the experimental cDNA sample prepared similarly.

Fig. 2-3 shows the log-log relationship between the amplified signal of the PCR product and the concentration of nucleic acids in the template of the positive control sample. As expected, the log-log relationship is linear between the template and the product.

**c. Optimum concentration of cDNA for samples and calculations :**

The level of each cytokine or gene message was expressed as a percentage of cyclophilin (Cyp). The amount of Cyp message should, in theory, be constant in each cell, and thereby serve as a internal control reflecting the number of cells, and the total amount of message remaining after total RNA extraction, and cDNA synthesis procedure. Fig 2-3 shows the amplification curves for Cyp and IFNγ for an example sample. Points representing three serial dilutions (A, B, and C since they lie on the exponential part of the amplification curve) were chosen for each gene and divided by the corresponding Cyp dilutions (a, b, and c). The following equation was used to calculate the value of each gene message of interest :

$$\frac{[((A \times dil) + (B \times dil) + (C \times dil))/min]}{[((a \times dil) + (b \times dil) + (c \times dil))/min]} \times 100$$

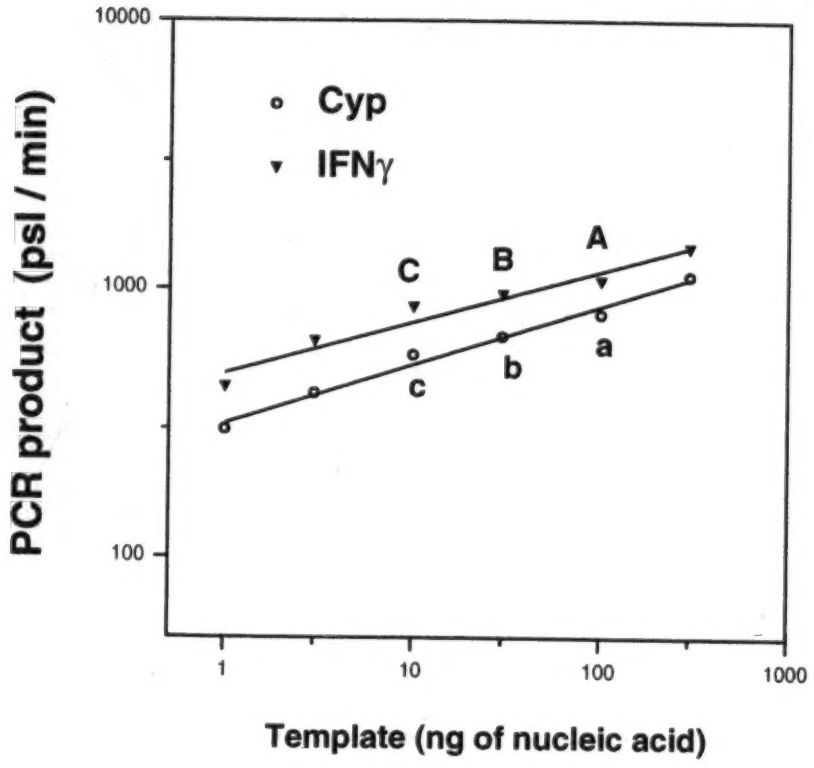



**Fig. 2-3:** Amplification of cyclophilin (Cyp) and interferon gamma (IFNγ) from serially diluted cDNA of an experimental sample. The PCR product is expressed in photo-stimulated luminescence/exposure time (psl/min). A,B, and C represent the dilutions 1/10, 1/30, and 1/90.

# Chapter 3

# The effect of complete Freund's adjuvant (CFA) on cytokine gene expression profiles in autoimmune diabetes-prone BB rats

# I. Introduction

Complete Freund's adjuvant (CFA) is a preparation of heat-killed *Mycobacterium tuberculosis* in mineral oil emulsion. CFA is used as an adjuvant to potentiate immune responses to antigens, e.g. in vaccination protocols. CFA acts as an immunostimulant because it induces inflammatory cytokines (e.g. IL-1, IL-6, and TNFα) due to the presence of lipopolysaccharides (LPS) in the bacterial wall.

## 1. CFA effect in the NOD mouse:

Singh and colleagues (177) first discovered that a single injection of young (5 weeks) NOD mice with CFA led to protection against the development of diabetes. Histological examination revealed that this protection was through substantial reduction of pancreatic insulitis. The levels of natural suppressor (NS) cells were discovered to be elevated in the spleens of CFA-treated mice, together with decreased splenic lymphocyte proliferative responses. These splenic NS cells were found to be Thy-1$^-$ radioresistant nonadherent cells that appeared to have originated from the bone marrow.

At the same time, another group (178) attempted the use of CFA as an adjuvant containing self-antigens (homogenates of pancreatic islets isolated from pre-diabetic NOD mice) with the intention of inducing the onset of diabetes prematurely in 8-9 wk-old male and

female NOD mice. To the investigators surprise, the results from those, and follow-up studies, showed that CFA conferred a state of protection against the development of diabetes in the CFA-treated animals. In addition, the incidence of histologically identifiable insulitis was also reduced in the CFA-treated mice by approximately 50%, compared to control ones, receiving no or saline injection. Upon further investigation the investigators reported failure of the splenic lymphocytes to respond to *in vitro* stimulation by the mitogen, Con-A, or by anti-CD3. They also reported the increase of splenic Thy-1$^-$ nonadherent cell populations (CD3$^-$, CD4$^-$, CD8$^-$, nonphagocytic, esterase negative, and Mac1$^+$) in the CFA-treated animals. These cells suppressed Con-A- or anti-CD3-induced proliferation of T-lymphocytes derived from either the spleen or thymus of untreated NOD mice. Interestingly, in the CFA-treated animals, the thymus- and lymph node-derived cells did not have the same diminished response to stimuli as did the spleen cells, suggesting that the suppressive effect of CFA was restricted to the spleen.

The role of CFA in the protection against diabetes was investigated further in syngeneic islet transplantation in diabetic NOD mice (179). When CFA was administered at the time of islet transplantation, the median survival time (MST) of CFA-treated syngeneic islet recipients was extended beyond 107 days compared to 11 days in control islet recipient mice. On the other hand, CFA treatment had no effect on extending the MST in NOD mice transplanted with allogeneic islets, suggesting different pathways for autoimmune destruction of islets from that of allograft immunity.

Another study (180) confirmed the effect of CFA treatment in the NOD mouse and indicated the importance of the timing of the CFA treatment in syngeneic islet transplantation into diabetic NOD mice. The study indicates that CFA should be administered after transplantation to confer protection against recurrent disease and not before. This is somewhat contradictory to findings of other investigators that, at least in primary autoimmune diabetes, CFA has to be administered early (i.e. at the time insulitis begins) to prevent the development of diabetes (see below).

**2. CFA Effect in the BB rat :**

CFA was also administered to the BB rat in an attempt to prevent diabetes (165). It was reported in that study that administration of CFA to the BB rat between 9 and 28 days of age reduced the incidence of diabetes at 120 days of age from 89% to 10-28%. Administration of CFA after the age of 40 days was ineffective. Incidentally, the insulitis starts to develop around the age of 30 days in the BB rat. In that study, however, the CFA treatment did not significantly decrease the infiltration of pancreatic islets by mononuclear leukocytes (insulitis). Also, CFA treatment had no effect on splenic cell subsets. CFA-induced prevention of diabetes in the DP-BB rats was reported to be associated with increased NS-cell activity in the spleen (181) as had been reported in NOD mice.

**3. The mechanism of CFA protection :**

The studies above discussed the effect of CFA administration on the development of IDDM and on cellular subsets in the spleen of the treated animals. However, the mechanism by which CFA induced splenic NS cells and the mechanism by which these cells conferred nonspecific protection was not investigated. The authors (165) (177) postulated that since CFA was known to induce the secretion of a variety of cytokines it was possible that CFA could exert its protective effects through the induction of cytokines over a critical period in diabetes development, and this, somehow, might lead to immune self-tolerance and circumvention of autoimmunity. One study (182) indicated that CFA may prevent the development of diabetes by restoring TNF production by macrophages from the diabetes-prone (DP)-BB rats (TNF production by peritoneal macrophages from DP-BB rats was deficient compared to that from macrophages of diabetes resistant (DR)- BB rats).

Subsequently, it was reported (183) that administration of CFA to NOD recipients of syngeneic islets prevented destruction of β-cells in the islet grafts; however insulitis was not prevented. Importantly, a relative increase in IFNγ-producing cells was observed in the untreated (control) mice, whereas the insulitis in the CFA-treated recipients was IL-4-rich (i.e. relative increase in IL-4-producing cells) and nondestructive, and β-cells were not destroyed, and as a result diabetes did not develop.

**4. Hypothesis:**

***In view of the Th1/Th2 paradigm and its possible role in the development of IDDM discussed previously in the introduction (Chapter 1, section VI), we hypothesized that the protective effect of CFA treatment against IDDM might be mediated through the downregulation of Th1 cytokines and/or upregulation of Th2 cytokines.***

**5. Study protocol :**

To test the hypothesis stated above, a single injection of CFA or PBS (100 µl i.p.) was administered to DP-BB rats at the age of 25 days. The experimental protocol is shown in Fig. 3-1. In the first study, diabetes incidence was followed until 120 days of age. The rats were monitored daily for glucosuria, from 50 days of age. Diabetes was defined as the presence of 4+ glucosuria (Tes-tape, Lilly Research Laboratories, CA) and a tail vein plasma glucose ≥ 11 mmol/l measured on a glucometer (Elite, Bayer, CA).

In the second study, CFA-and PBS-treated DP-BB rats were anesthetized with pentobarbital (50 mg/kg of body weight) between the ages of 60-75 days (when incidence of diabetes is approximately 25-50%). Pancreases and spleens were excised for histological examination, insulin content assay, mononuclear leukocyte subset analyses, and cytokine mRNA analyses as described in chapter 2.

Figure 3-1:
Protocol for CFA
Administration Studies

# II. Results

## 1. Diabetes incidence :

No significant changes were observed in the gain of body weight in DP-BB rats treated with CFA (data not shown). The effect of CFA treatment on the incidence of diabetes is shown in Fig. 3-2. Diabetes starts to develop in the DP-BB rats injected with PBS (n=12), known as the control group, at the age of 48 days and the incidence of the disease reaches 92% by the age of 120 days. The incidence of diabetes in DP-BB rats treated with CFA (n=17), on the other hand, began at the age of 60 days and reached 50% by the age of 120 days. Therefore, CFA treatment delayed and reduced the incidence of diabetes by approximately 50% by the age of 120 days in DP-BB rats.

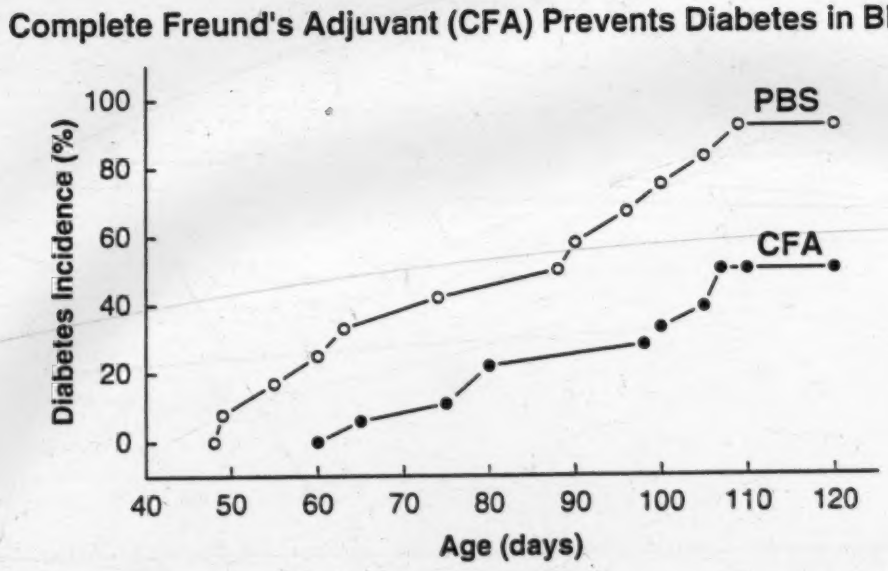



**Fig. 3-2:** BB diabetes-prone (DP) rats were injected i.p. with PBS (o, n=12) or with CFA (•, n=18) at age 25d. Diabetes incidence at 65d in CFA-injected DP-BB rats (1 of 17, 6%) was significantly less than in PBS-injected DP-BB rats (4 of 12, 33%), $p < 0.05$ by Chi-squared test, 2x2 tables, df.=1. Diabetes incidence at 120 days in CFA-injected DP-BB rats (9 of 18, 50%) was significantly less than in PBS-injected DP-BB rats (11 of 12., 92%), $p < 0.05$ by Chi-squared test, 2x2 tables, df.=1.

**2. Pancreatic histology, insulitis, and insulin content :**

The effect of CFA treatment on the degree of insulitis could be seen from the histological staining of the islet tissues in Fig. 3-3 and from the insulitis score shown in Table 3-1. Fig. 3-3, panel A shows an islet of a DR-BB rat, where the insulitis is absent with grade of 0.0 ± 0.0 for the insulitis score (Table 3-1). In the DP-BB rat group, the infiltrating leukocytes invaded the islet as seen in Fig. 3-3 panel B (prediabetic, DP-PBS(PD), insulitis score of 1.3 ± 0.1). The destruction of the islets, inferred from the lack of insulin production (Fig. 3-3 panel D, acutely-diabetic, DP-PBS(AD)) and insulin content value of 3.5 ± 0.6 (Table 3-1) was associated with an increase in the insulitis score (from 1.3 ± 0.1 to 2.8 ± 0.2, Table 3-1).

Although, the treatment of DP-BB rats with CFA was associated with reduction of insulitis from 2.1 ± 0.3 in the control group (DP-PBS) to 0.8 ± 0.1 in DP-CFA group (Table 3-1), the difference between these groups was not statistically significant. However, table 3-2 shows that the DP-CFA group has significantly less mononuclear leukocytes/islet (274.2 ± 14.9) than the DP-PBS group (1,407.4 ± 228.3, $p < 0.05$).

Closer examination of the DP-PBS control group shows that under the protection of CFA the infiltrating lymphocytes were not prevented from reaching the islets (Fig. 3-3, panel C) with insulitis score of 0.8 ± 0.1 (Table 3-1). This indicates that the amount of mononuclear leukocyte infiltrate in the pancreatic islets of DP-CFA

group is similar to that of DP-PBS control group before they became acutely-diabetic (DP-PBS(PD), insulitis score of 1.3 ± 0.1). This can also be seen in Table 3-2, where the total number of mononuclear leukocytes/islet of DP-CFA group is similar that of the DP-PBS(PD) group. However, insulitis in the DP-CFA group is significantly less than that in the acutely-diabetic DP-PBS(AD) group ($p < 0.05$). Also, the total number of mononuclear leukocytes/islet of DP-CFA group is significantly less than that of the acutely-diabetic DP-PBS(AD) group ($p < 0.001$). It is important to note that none of the DP-CFA rats were acutely-diabetic until at the time they were sacrificed for these experiments. Finally, Table 3-1 shows that the total pancreatic β-cell mass (pancreatic insulin content) in the different groups of BB rats was inversely related to the insulitis score in those groups.

**In conclusion, CFA treatment of DP-BB rats delayed the onset and decreased the frequency of diabetes development. CFA treatment did not prevent the initial infiltration of islets by mononuclear leukocytes. It significantly reduced the total number of mononuclear leukocytes/islet compared to the control group. However, the insulitis and total number of mononuclear leukocytes/islet are similar in the DP-CFA and the DP-PBS(PD) groups. Therefore, CFA appeared to delay and reduce the development of IDDM by preventing the transformation of the islet infiltrate into the aggressive and destructive insulitis (usually seen during acute-diabetes) to**

the insulin-producing β-cells in the islets by unknown mechanisms.

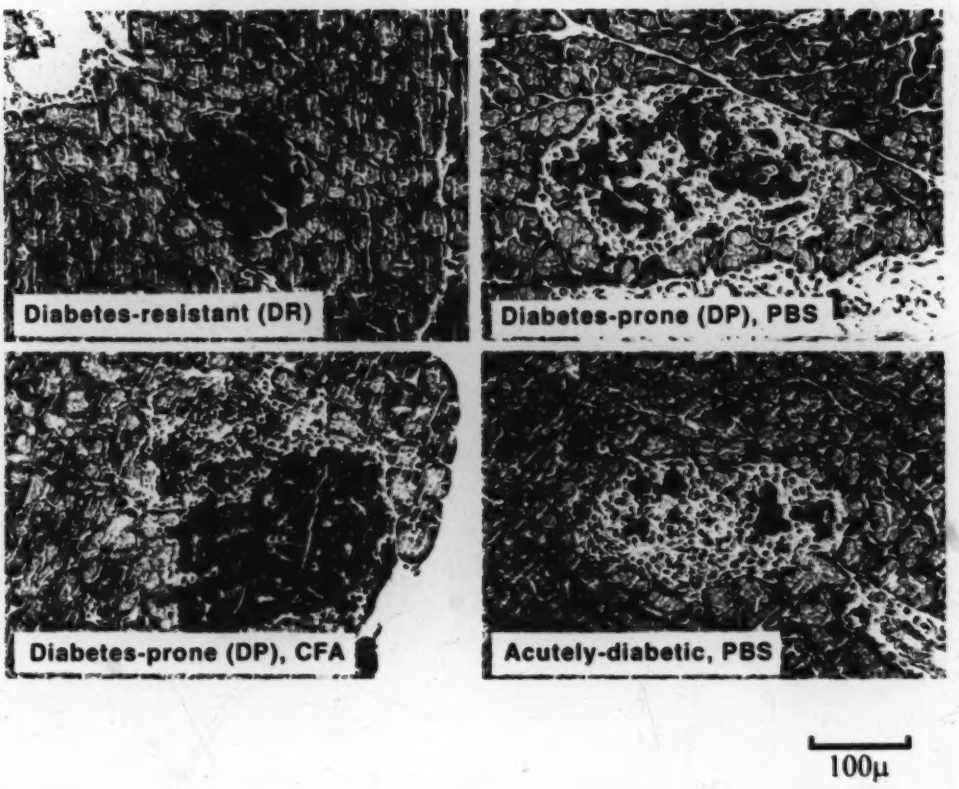



**Fig. 3-3** : Photomicrographs of pancreatic sections from 60- to 75-day-old BB rats. The sections are stained with anti-insulin serum to reveal β-cells (brown stain) and counterstained with hematoxylin. panel A: DR rats with intact islet β-cells and few surrounding mononuclear leukocytes. panel B: DP-BB rat pancreas showing islet infiltration by mononuclear leukocytes and some reduction of the β-cell mass. panel C: CFA-treated DP-BB rat pancreas with peri-islet accumulations of mononuclear leukocytes and intact islet β-cells. panel D: acutely diabetic rat pancreas with extensive islet infiltration by mononuclear leukocytes and substantial reduction of the β-cell mass.

# Table 3-1

**Pancreatic islet histology and insulin content in diabetes-prone rats treated with complete Freund's adjuvant (CFA)**

| BB rat group at age 65 days | *n* | Blood glucose (mmol/l) | Insulitis score (grade 0-4) | Insulin content (ng/mg pancreas) |
|---|---|---|---|---|
| DR | 6 | 5.5 ± 0.3 | 0.0 ± 0.0 | 49.7 ± 3.0 |
| DP-PBS | 12 | 11.9 ± 1.7 | 2.1 ± 0.3 | 21.1 ± 5.4 |
| DP-CFA | 6 | 6.9 ± 0.4 | 0.8 ± 0.1 | 43.0 ± 2.0 |
| DP-PBS (PD) | 6 | 6.3 ± 0.6 | 1.3 ± 0.1 | 38.7 ± 1.5 |
| DP-PBS (AD) | 6 | 17.5 ± 0.5 | 2.8 ± 0.2* | 3.5 ± 0.6* |

Diabetes-prone (DP) BB rats were injected i.p. with PBS (DP-PBS) or with CFA (100 µl CFA/50g body weight) at the age of 25 days (DP-CFA). At age 60-75 days, the blood glucose values were obtained, pancreases were removed for determination of insulin content (ng/mg pancreas).

Data are means ± SE for the number of rats (n) indicated for the following groups: DR = diabetes-resistant BB rats, DP-control = diabetes-prone BB rats injected with PBS, and DP-CFA = diabetes-prone BB rats injected with CFA. The DP-PBS is also shown as broken down into DP-PBS(PD) = prediabetic diabetes-prone BB rats, and DP-PBS (AD) acutely-diabetic diabetes-prone BB rats.

Histological examination revealed leukocytes infiltrating the islets in the DP-control and DP-CFA groups. Islet inflammation (insulitis) was graded from 0 to 4 according to the extent of inflammation: 0 = none, 1 = only peri-islet infiltration, 2 = < 25% islet area infiltrated, 3 = 25-75% islet area infiltrated, and 4 = > 75% islet area infiltrated. The slides were read and score reported in a double-blinded fashion.

The asterisks denote statistical significance compared with the DP-CFA group (* = $p < 0.05$) using the Kruskal-Wallis non-parametric ANOVA followed by the Dunn's multiple comparison test (for comprehensive statistical comparisons see text).

**3. Isolation of mononuclear leukocytes from pancreatic islets :**

Differences in degrees of insulitis suggested by the photomicrographs shown in Fig. 3-3 were more completely quantitated in the experiments shown in Table 3-2. Fewer islets were obtained from the acutely-diabetic rats (Table 3-2), as expected from the destruction of islets by infiltrating leukocytes in these rats (Fig. 3-3, panel D). However, the islets that were isolated from diabetic rats contained significantly more mononuclear leukocytes than the DP-PBS(PD) and DP-CFA rats ($p < 0.0001$), and the islets of DR-BB rats contained the least (Table 3-2). In these experiments, statistical significance was found between the number of total number of mononuclear leukocytes in the DP-PBS control group and the DP-CFA group ($p < 0.01$). This is due to the fact that the number of rats in each group is considerably more than the groups used in the experiments of table 3-1. It is important to note that none of the DP-CFA rats were acutely-diabetic until they were sacrificed for these experiments as was reported for the experiments in table 3-1.

The purity of mononuclear leukocytes isolated from the four groups ranged from a mean of 68% to 83%. Again, the high number of rats in each group from the experiments of table 3-2 helped find statistical significance ($p < 0.0001$) between the percentages of OX1 positive cells isolated from the islets of the DP-CFA group and those isolated from the islets of the DP-PBS group. This is largely due to

the contribution of the DP-PBS(AD) group, which always produced the highest percentage of OX1 positive cells. This can be explained by reasoning that during acute-diabetes, the majority of islet mass is absent which results more efficient isolation of islet-associated mononuclear leukocytes.

# Table 3-2

## Mononuclear leukocytes isolated from islets of BB rats

| BB rat group | Treatment | *n* | Islets: no. per pancreas | Mononuclear leukocytes: Total no. | Mononuclear leukocytes: No. per islet | Mononuclear leukocytes: % OX1* |
|---|---|---|---|---|---|---|
| DR | PBS | 16 | 496.0 ± 19.0 | 47,625.0 ± 4,493.0 | 98.0 ± 9.7 | 77.0 ± 2.2 |
| DP | PBS | 38 | 287.1 ± 25.0* | 234,130.0 ± 17,455.0** | 1,407.4 ± 228.3* | 78.2 ± 1.2*** |
| DP | CFA | 17 | 426.0 ± 14.4 | 114,750.1 ± 4,967.8 | 274.2 ± 14.9 | 69.5 ± 1.5 |
| DP | PBS (PD) | 18 | 424.2 ± 23.4 | 128,944.0 ± 6,898.2 | 317.3 ± 23.4 | 75.2 ± 1.9 |
| DP | PBS (AD) | 20 | 164.0 ± 13.3*** | 328,800.0 ± 9,657.8*** | 2,388.5 ± 292.4*** | 81.0 ± 1.2*** |

Diabetes-prone (DP) BB rats were injected i.p. with PBS (DP-PBS) or with CFA (100 µl CFA/50g body weight) at the age of 25 days (DP-CFA). At age 60-75 days pancreases were removed, islets were isolated and mononuclear leukocytes were separated from the islets; their purity was determined by staining with a monoclonal antibody (OX1) to rat leukocytes. Leukocytes obtained from the islets of each rat were studied separately.

Data are means ± SE for the number of rats (n) indicated for the following groups: DR = diabetes-resistant BB rats, DP-control = diabetes-prone BB rats injected with PBS, and DP-CFA = diabetes-prone BB rats injected with CFA. The DP-PBS is also shown as broken down into DP-PBS(PD) = prediabetic diabetes-prone BB rats, and DP-PBS (AD) acutely-diabetic diabetes-prone BB rats.

The asterisks denote statistical significance compared with the DP-CFA group (* = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$) using the Kruskal-Wallis non-parametric ANOVA followed by the Dunn's multiple comparison test (for comprehensive statistical comparisons see text).

**4. Splenic mononuclear leukocyte subsets :**

Table 3-3 shows that DP-BB rats had a pan-T-Cell (CD5) lymphopenia (decreased $OX19^+$ T-cells) compared to DR-BB rats, and the decreases in T-cells consisted of a decrease in both $CD4^+$ T-cells ($W3/25^+$) T-cells and $CD8^+$ T-cells, as previously reported for DP-BB versus DR-BB rats. CFA treatment of DP-BB rats did not change any splenic cell subset compared to control, PBS-treated DP-BB rats (Table 3-3).

# Table 3-3

**Monoclonal antibody-defined splenic lymphoid cell subsets of BB/Wor rats treated with CFA[a]**

| BB rat group | Tissue | Mononuclear leukocyte subset (% of total leukocytes)[b] | | | | |
|---|---|---|---|---|---|---|
| | | CD5 (OX19⁺) | CD4 (W3/25⁺) | CD8 (OX8⁺) | NK (3.2.3⁺) | Mϕ (OX42⁺) |
| DR | Spleen | 50.0 ± 4.5 | 38.7 ± 5.0 | 28.0 ± 3.0 | 4.0 ± 1.0 | 18.0 ± 2.0 |
| DP, control | Spleen | 20.0 ± 3.0 | 23.4 ± 1.0 | 33.0 ± 4.0 | 19.2 ± 2.0 | 20.0 ± 2.0 |
| DP, CFA | Spleen | 22.0 ± 4.0 | 20.2 ± 2.0 | 35.0 ± 3.0 | 17.0 ± 1.0 | 21.0 ± 1.0 |

[a] Diabetes-prone (DP) BB rats were injected i.p. at age 25 days with saline (100 $\mu$l) or CFA (100 $\mu$l). DR are diabetes-resistant BB rats.

[b] Percentage of cells staining positive with the different lymphoid cell subset markers are shown as mean values ± SE for six rats from each group at age 65-75 days.

**5. Cyclophilin steady-state mRNA levels are proportional to numbers of cells submitted to RT-PCR analysis:**

Because samples submitted to RT-PCR analysis contained different numbers of mononuclear leukocytes, it was necessary to demonstrate that mRNA level (PCR product) for cyclophilin, chosen as a house-keeping gene (i.e. constant expression from cell to cell), was proportional to the number of cells in the sample. Fig. 3-4, panel C shows that cyclophilin mRNA expression correlated positively with numbers of islet-infiltrating mononuclear leukocytes (regression coefficient, r= 0.89, $p < 0.0001$). Therefore, cytokine mRNA levels (PCR product) measured in different samples could be compared after normalizing to cyclophilin mRNA levels in the respective samples (as shown in the equation in Chapter 2, section V).

**The relationship between Cyclophilin (Cyp) mRNA expression and the number of islet mononuclear Cells (IMNC's) isolated from the islets of BB rats**

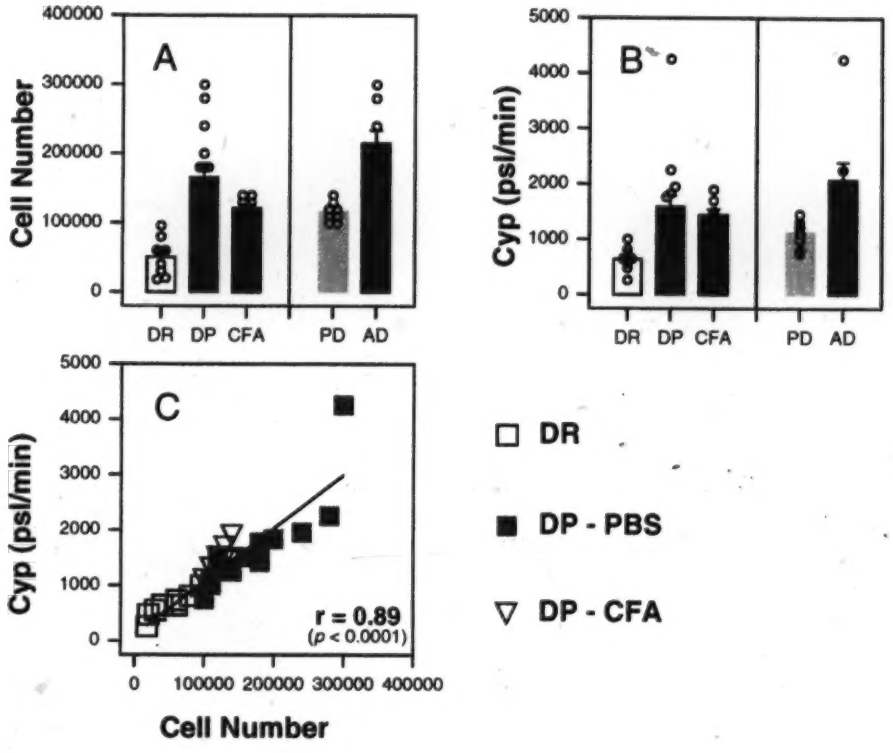



**Fig. 3-4:** The total number of islet mononuclear cells (IMNC's), panel A, and Cyclophilin mRNA levels (Cyp), panel B, and the relationship between them, panel C, in islets of BB rats, ages 60-75 days. DR rats (n=8, white) were injected at age 25 days with PBS. DP-BB rats were injected at age 25 days with saline (DP-PBS, n=16, red) or CFA (DP-CFA, n=8, blue). 50% of the DP-PBS rats were prediabetic (PD, n=8, yellow) at the time of sacrifice, whereas the rest were acutely diabetic (AD, brown). AD rats were studied within 2 days of diabetes onset. Cyp PCR levels (psl/min) are shown for each group, panel B. Individual animals in each group are shown and mean values for the groups are indicated by the bars + SE.

**6. Cytokine steady-state mRNA levels in islet mononuclear leukocytes :**

Cytokine steady-state mRNA levels in mononuclear leukocytes isolated from islets of the BB rat groups are shown in Fig. 3-5. IFNγ steady-state mRNA levels were significantly higher in mononuclear leukocytes isolated from islets of the DP-PBS group than in those of the DR group ($p < 0.001$), and the DP-CFA group ($p < 0.05$). The DP-CFA group had similar levels to those of the DR group. The levels of IL-2 and IL-10 steady-state mRNA were also higher in mononuclear leukocytes isolated from islets of the DP-PBS group than in those of the DR group; however, the differences did not reach statistical significance.

The levels of IL-4 steady-state mRNA did not show any changes among the groups. The levels of TNFα mRNA were reduced in mononuclear leukocytes isolated from islets of the DP-PBS group compared with those of the DR or DP-CFA groups. Furthermore, the difference between the DP-PBS and DP-CFA groups reached statistical significance ($p < 0.05$). No significant differences were seen in the levels of any cytokine mRNA between prediabetic (PD) and acutely-diabetic (AD) subgroups of the DP-PBS rats.

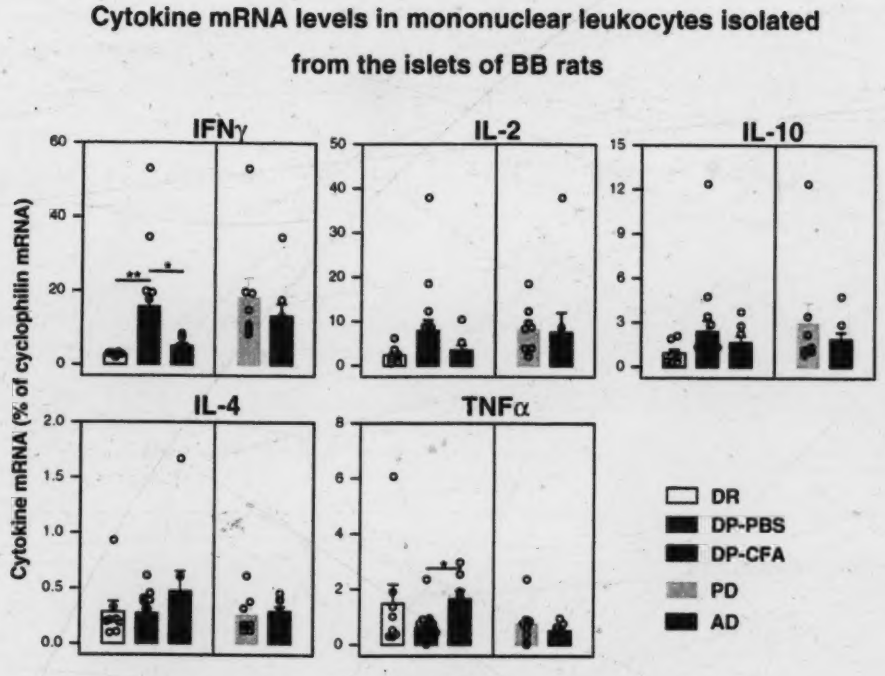



**Fig. 3-5:** Cytokine steady-state mRNA levels in mononuclear leukocytes isolated from islets of BB rats, ages 60-75 days. DR rats (white, n=8) were injected at age 25 days with PBS. DP-BB rats were injected at age 25 days with saline (DP-PBS, red, n=16) or CFA (DP-CFA, blue, n=8). Diabetes-resistant (DR) rats (n=8, white) were injected at age 25 days with PBS. 50% of the DP-PBS rats were prediabetic (PD, yellow, n=8) at the time of sacrifice, whereas the rest were acutely diabetic (AD, brown, n=8). AD rats were studied within 2 days of diabetes onset. Levels of cytokine mRNA (PCR product) are expressed as a percentage of cyclophilin (Cyp) mRNA. Values for individual animals in each group are shown as open circles (o). Statistical significance was determined by a non-parametric ANOVA test (Kruskal-Wallis). * $p < 0.05$, ** $p < 0.01$ vs. DP-PBS group.

There were also significant correlations between steady-state mRNA levels of IFNγ and IL-2, and IFNγ and IL-10, in individual animals involved in the experiment. Fig. 3-6, panel A shows the significant positive correlation ($r= 0.76$, $p < 0.0001$) found between the levels of IFNγ and IL-2 steady-state mRNA levels in the BB rats groups, while Fig. 3-6, panel B shows an even greater positive correlation ($r= 0.84$, $p < 0.0001$) between IFNγ and IL-10 mRNA levels in the same BB rats.

**Correlations between IFNγ, IL-2, and IL-10 mRNA levels in mononuclear leukocytes from islets of individual BB rats**

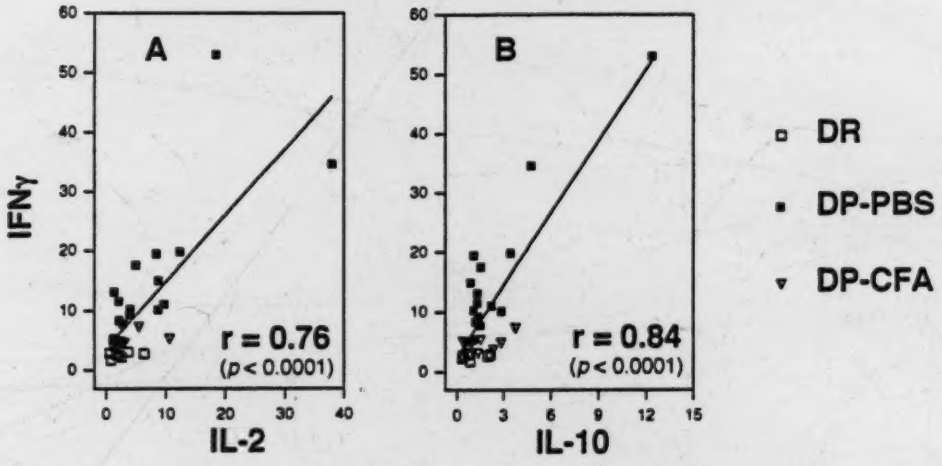



**Fig. 3-6:** Correlations between IFNγ and IL-2 (A), and between IFNγ and IL-10 (B) steady-state mRNA levels in mononuclear leukocytes isolated from islets of individual BB rats: DR (n=8), DP-PBS (n=16) and DP-CFA (n=8) for animals from the studies as described in the legend of Fig. 3-5. The correlation coefficients (r=0.76 for A ($p < 0.0001$), and r=0.86 for B ($p < 0.0001$)) were calculated using SigmaPlot v4.11 (Jandel Scientific).

**7. Cytokine steady-state mRNA levels in spleens :**

Fig. 3-7 shows cytokine expression profiles in spleens of BB rats from the different groups. The level of IFNγ steady-state mRNA was significantly decreased ($p < 0.05$) in the spleens DP-BB rats treated with CFA compared with that in the spleens of the DP-PBS group. A closer look at the breakdown of the latter group indicates that the expression of IFNγ steady-state mRNA is more pronounced in the spleen after the onset of diabetes (DP-PBS(AD) group). The level of IL-10 steady-state mRNA, on the other hand, was significantly increased in the DP-CFA group compared with the DP-BB group; also IL-10 steady-state mRNA was significantly lower in the DP-PBS group than in the DR-BB group.

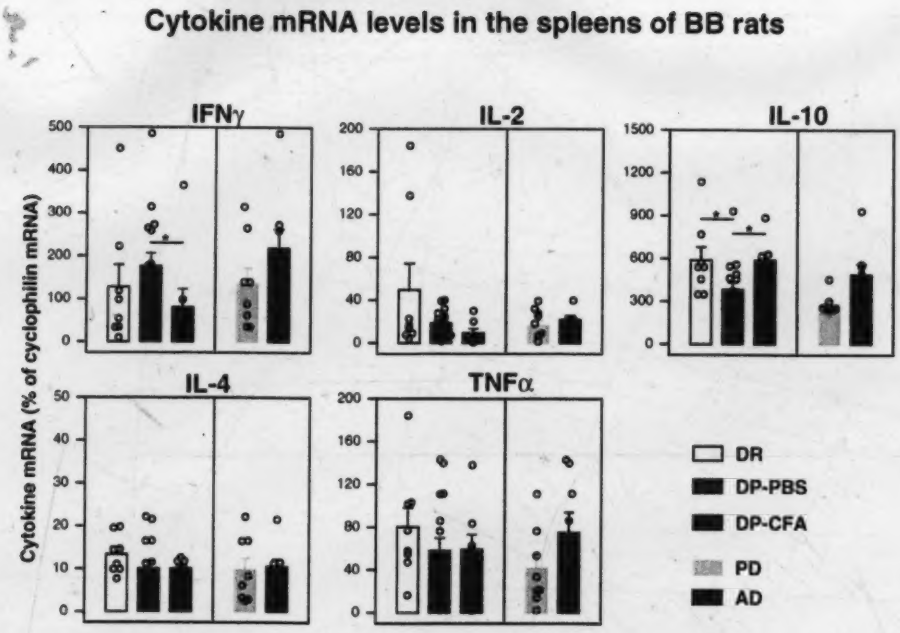



Fig. 3-7: Cytokine steady-state mRNA levels in spleens isolated from BB rats, ages 60-75 days. DR rats (white, n=8) were injected at age 25 days with PBS. DP-BB rats were injected at age 25 days with saline (DP-PBS, red, n=16) or CFA (DP-CFA, blue, n=8). 50% of the DP-PBS rats were prediabetic (PD, yellow, n=8) at the time of sacrifice, whereas the rest were acutely diabetic (AD, darker red, n=8). AD rats were studied within 2 days of diabetes onset. Levels of cytokine mRNA (PCR product) are expressed as a percentage of cyclophilin (Cyp) mRNA. Values for individual animals in each group are shown as open circles (o). Statistical significance was determined by a non-parametric ANOVA test (Kruskal-Wallis). * $p < 0.05$ vs. DP-PBS group.

# III. Discussion

Two possible mechanisms may be responsible for the protective effect of CFA against diabetes development in the DP-BB rat: 1) CFA could prevent inflammation of the pancreatic islets, 2) CFA could allow the inflammation of the islets (insulitis) but interfere with the islet-β-cell destructive process and the resultant IDDM.

Histological examination of pancreatic islets revealed that the first possibility was not responsible for the protective effect of CFA. The insulitis score (Table 3-1), and the total number of mononuclear cells isolated from the islets (Table 3-2), indicate that CFA treatment was not able to prevent the initial invasion of the islets by the infiltrating cells nor the formation of insulitis similar to that of the control group before acute-diabetes (DP-PBS(PD)). However, it prevented the transformation of this mild insulitis during prediabetes (Fig. 3-3, panel B, and Table 3-2 DP-PBS(PD) group) into the aggressive insulitis observed during acute-diabetes (Fig. 3-3, panel D, and Table 3-2 DP-PBS(AD) group). Therefore, it appears that CFA protected against diabetes by preventing the islet-infiltrating leukocytes from being destructive to the islet β-cells. The mechanisms by which CFA protects the islet-β-cells from destruction and preserves their ability to produce insulin (Fig. 3-3, panel C) may be explained by the cytokines produced by the infiltrating cells.

Previous studies in NOD mice (183) (184) have reported that the type 1 cytokine, IFNγ, is predominately expressed in what was termed destructive insulitis, whereas benign or non-destructive insulitis observed with CFA administration was rich in the type 2 cytokine, IL-4. Therefore, I hypothesized that the protective effect of CFA against diabetes development might be mediated by a qualitative change in the insulitis lesion consisting of a decrease in expression of type 1 cytokines and/or an increase in expression of type 2 cytokines.

I found that treatment of DP-BB rats with CFA, which protected against islet-β-cell destructive insulitis, downregulated both IFNγ (significantly) and IL-2 mRNA steady-state levels in islet-infiltrating mononuclear leukocytes (Fig. 3-5). Furthermore, the significant correlation between IFNγ and IL-2 steady-state mRNA levels (Fig. 3-6, panel A) in mononuclear cells from islets of individual animals is indicative of a possible common cell source, or at least common regulation of expression, for both cytokines. Because IFNγ and IL-2 are the cytokines that characterize Th1 cells, these findings suggest that Th1 cells infiltrating the pancreatic islets may promote β-cell destruction and consequently IDDM. This suggestion is supported by several studies reported in the literature. For example, the destructive role of IFNγ in the development of diabetes was deduced from studies where deletion of IFNγ (104) or its receptor (IFNγR) (105), from diabetes-prone mice, resulted in a delay in the onset and reduction in the frequency of IDDM in the former and complete prevention of IDDM in the latter. In addition, administration of anti-

IFNγ antibodies to diabetes-prone animals (mice (108) (109) and rats (107)) also resulted in prevention of diabetes. Furthermore, administration of anti-IL-2 receptor monoclonal antibody also resulted in suppression of diabetes in mice (111).

Contrary to my hypothesis, however, the protection against IDDM, conferred by CFA, did not correlate with increased expression of type 2 cytokines. Thus, IL-4 steady-state mRNA level was not significantly increased after CFA treatment (Fig. 3-5). Also, IL-10 steady-state mRNA level was downregulated, albeit lacking statistical significance, after CFA treatment. In addition, there was a significant positive correlation between IFNγ and IL-10 steady-state mRNA levels in mononuclear cells from islets of individual animals (Fig. 3-6). The interpretation of this finding may be that : 1) IL-10 is acting as a pro-inflammatory cytokine instead of a type 2 suppressive cytokine, or 2) IL-10 is actually downregulating IFNγ expression and the latter would be even higher if IL-10 expression was not increased. Recent literature suggests that IL-10 may play a pro-inflammatory role in the development of diabetes since transgenic expression of IL-10 by islet β-cells in the NOD mouse accelerated diabetes onset (96) (97).

To test whether the effect of CFA on cytokine mRNA expression was localized in the pancreatic islets only, cytokine expression was also investigated in the spleen. CFA significantly downregulated IFNγ steady-state mRNA level in the spleen (Fig. 3-7), as had been observed in the islets (Fig. 3-5). In contrast, IL-10 steady-state

mRNA level was significantly upregulated in the spleens of the CFA-treated rats, whereas IL-10 steady-state mRNA levels were decreased in the islets of the CFA-treated rats. These findings suggest a possible role for systemic IL-10 in the protection against diabetes development. Indeed, systemic administration of IL-10 has been reported to protect against diabetes development in NOD mice (99) (100).

Although the present study does not identify the cell source of the cytokines detected in the islets or spleen, it shows the predominance of certain cytokine messages in the islets or the spleens during spontaneous disease (DP-PBS) and during the protection conferred naturally (DR) or with CFA administration. This study indicates that CFA administration may provide protection against the development of diabetes through the downregulation of type 1 cytokines (IFNγ) locally in the islets and systemically (i.e. spleen).

Further investigation of the role of IL-10 in the development of autoimmune diabetes should be carried out in animals deficient in the production of this cytokine (i.e. IL-10 knock-out mutant). Also, administration of monoclonal antibodies directed against IL-10 may help elucidate the role of this cytokine in IDDM.

The effect of CFA treatment to increase TNFα steady-state mRNA level in the insulitis lesion was also unexpected. Although TNFα is generally considered a pro-inflammatory cytokine, there is

evidence to support its role as a protective cytokine in the development of diabetes. Unlike IFN(α/γ) transgenic mice which develop diabetes even in diabetes-resistant strains (89), transgenic expression of TNFα by islet β-cells (185) was found to promote massive insulitis, rich in both $CD4^+$ and $CD8^+$ T-cells as well as macrophages, but not diabetes. Furthermore, transgenic expression of TNFα by β-cells in NOD mice protected against diabetes development (92). In other studies, treatment of NOD mice (older than 4 weeks of age) with TNFα resulted in a delay in the onset of diabetes (101). Also, TNFα treatment of DP-BB rats decreases the incidence of diabetes (186) and TNFα antiserum abrogates the protective effect of CFA against diabetes development in DP-BB rats (187). Therefore, the present study showing that the expression of TNFα in the insulitis lesion correlates with CFA protection against diabetes supports those studies that identify a regulatory role for TNFα in the islets β-cell directed autoimmune response. TNFα may prevent autoimmune destruction of β-cells by affecting APC function in a manner that blocks initiation of the autoimmune response; alternatively TNFα may delete autoreactive T-cells by apoptosis (188).

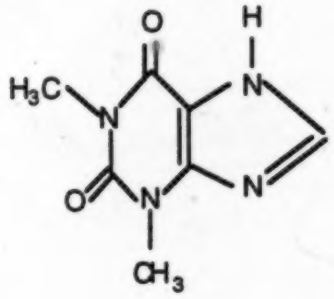
# Chapter 4

## The effect of theophylline on cytokine gene expression profiles in autoimmune diabetes-prone BB rats

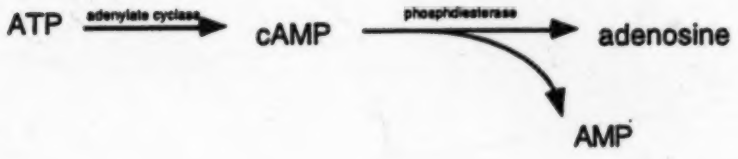
# I. Introduction

## 1. Theophylline structure and function :

Theophylline (Theo) is a member of the methylxanthine family which also includes caffeine and theobromine. Theo, 1,3-dimethylxanthine (chemical structure shown below), is a more potent phosphodiesterase inhibitor than caffeine or theobromine:




Theo functions as a non-selective phosphodiesterase inhibitor, i.e. the membrane-bound 3',5'-cyclic nucleotide phosphodiesterase which converts cAMP into AMP and adenosine is inhibited by Theo (189).



This leads to accumulation of intracellular cAMP which is believed to mediate the immunosuppressive effects of Theo. Pharmacological studies (190) indicated a role for cAMP in the differentiation of immature lymphocytes, whereas elevated levels of cAMP were associated with inhibition of mature differentiated lymphocyte functions. Furthermore, an increase of cAMP was related to inhibition of lymphocyte cytotoxicity (191).

## 2. Theo and IDDM :

It was reported that Theo could be used as an immunosuppressive agent to prolong allograft survival in diabetic rats (192). In that study, oral administration of Theo led to prolonging of the allograft of whole pancreas from Brown Norway rats into Lewis rats from 7 days in the control group to 11 days in the experimental group receiving Theo.

The effect of Theo administration on type 1 diabetic patients, with recent onset, was also investigated (193). The majority (70%) of theophylline-treated patients achieved remission (defined as an insulin requirement of less than 0.25 U/kg/d with a good metabolic control) whereas only 37.5% of the placebo-treated group achieved remission. The patients in the study were followed (i.e. follow-up period) for 16-32 months.

Theo also protected against diabetes induced by multiple low-dose streptozotocin in rats (194), and against spontaneous autoimmune diabetes in BB rats (166). The diabetes incidence in DP-BB rats treated with Theo in a fixed dose of 2 mg/ml in the drinking water resulted in the delay and reduction of diabetes incidence by approximately 40 % in the treated rats compared with their control littermates.

**3. Hypothesis :**

***Since Theo is considered an immunosuppressive agent and has been able to protect, however moderately, against the development of diabetes, we hypothesized that it may be doing so by downregulating Th1 lymphocyte function, and consequently type 1 cytokines, either directly or by upregulating Th2 lymphocytes and the production of type 2 cytokines which are known to downregulate type 1 cytokines.***

**4. Study protocol :**

To test this hypothesis, Theo was administered to DP-BB rats starting at the age of 25 days in their drinking water at a fixed dose (2 mg/ml), and the drinking water level was checked every morning to ensure water consumption. This dose has successfully been used before (166) to protect against diabetes. It produced serum Theo concentrations of 51.8 ± 3.0 µg/ml in DP-BB rats at 60 days, 20.0 ±

2.0 at 100 days. Theophylline levels decreased with increasing age (and body weight of the rat).

The experimental protocol is shown in Fig. 4-1. In the first study, diabetes incidence was followed until 100 days of age. The rats were monitored daily for glucosuria, from 50 days of age. Diabetes was defined as the presence of 4+ glucosuria (Tes-tape, Lilly Research Laboratories, CA) and a tail vein plasma glucose ≥ 11 mmol/l measured on a glucometer (Elite, Bayer, CA). In the second study, Theo-treated and control rats (receiving the water vehicle) were anesthetized with pentobarbital (50 mg/kg of body weight) between the ages of 60-75 days (when incidence of diabetes is approximately 25-50%). Pancreases were excised for histological examination, insulin content assay, mononuclear leukocyte analyses, and cytokine mRNA analyses as described in chapter 2.

Figure 4-1:
Protocol for Theophylline
Administration Studies

# II. Results

## 1. Diabetes incidence :

The effect of Theo treatment on the incidence of diabetes is shown in Fig. 4-2. Diabetes started to develop in the DP-BB rats (control group) at the age of 60 days and the incidence of the disease reached 95% by the age of 100 days. The incidence of diabetes in DP-BB rats treated with Theo (n=17) also began at the age of 60 days and reached 50% by the age of 100 days. Therefore, Theo treatment reduced the incidence of diabetes in the DP-BB rats by 40% by the age of 100 days.

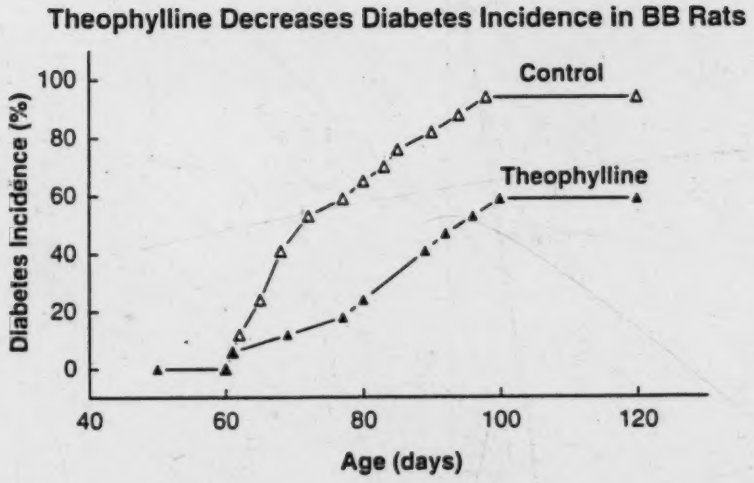



**Fig. 4-2:** BB diabetes-prone (DP) rats received theophylline (2mg/ml drinking water) or water (control) starting at age 25d. Diabetes incidence started at age 60d in both groups of DP-BB rats, reaching 60% in the control group and only 20% in the Theo -treated group by age 80d. At the end of the study, at 100 d, the incidence of diabetes in the control group reached 95%, while it was significantly less (60%) in the Theo-treated group ($p < 0.05$ by Chi-squared test, 2x2 tables, df.=1).

**2. Pancreatic histology, insulitis, and insulin content :**

The effect of Theo treatment on the degree of insulitis could be seen from the histological staining of the islet tissues (Fig. 4-3) and from the insulitis scores (Table 4-1). Fig. 4-3, panel A shows an islet of a prediabetic DP-control rat : leukocytes invaded the islet with an insulitis score of 2.7 ± 0.3 (Table 4-1) for DP-control(PD) rats and total number of mononuclear leukocytes/islet of 474 ± 89. Upon the onset of IDDM, this insulitis increased in the acutely-diabetic group to 3.9 ± 0.1 and total number of mononuclear leukocytes/islet of 5,480 ± 580 (PD-control (AD), Table 4-1). This increase in insulitis score was accompanied by lack of insulin production (insulin content of only 2.3 ± 0.6, Table 4-2). Fig. 4-3, panel B shows the insulitis under the protection provided by Theo treatment: leukocytes were not prevented from reaching the islets and the insulitis score was 2.6 ± 0.2 and the total number of mononuclear leukocytes/islet was 383 ± 20.

Theo treatment did not result in significant reduction of insulitis or the total number of mononuclear leukocytes/islet in the DP-Theo group (2.6 ± 0.2, Table 4-1) compared with the DP-control group (3.3 ± 0.2, Table 4-1). The amount of mononuclear leukocyte infiltrate in the pancreatic islets of DP-Theo group is similar to that of DP-control group before they became acutely diabetic (i.e. DP-PBS(PD), Table 4-1). It is important to note that none of the DP-Theo rats were acutely-diabetic until they were sacrificed for these experiments in table 4-1. In addition, Table 4-2 shows that the total pancreatic β-

cell mass (pancreatic insulin content) in the different groups of BB rats was inversely related to the insulitis score in those groups (Table 4-1).

**In conclusion, Theo treatment of DP-BB rats decreased the frequency of diabetes development; however Theo did neither prevent the initial infiltration of islets by mononuclear leukocytes nor reduce the amount of insulitis significantly. Therefore, the moderate reduction of diabetes incidence obtained with Theo treatment is due to other mechanisms.**

# Table 4-1

## Mononuclear leukocytes isolated from islets of BB rats

| BB rat group | *n* | Insulitis score (grade 0-4) | Islets: no. per pancreas | Mononuclear leukocytes: Total no. | Mononuclear leukocytes: no. per islet | Mononuclear leukocytes: % OX1* |
|---|---|---|---|---|---|---|
| DR | 6 | 1.0 ± 0.1 | 513 ± 10 | 58,000 ± 9,320 | 113 ± 17 | 82.1 ± 1.0 |
| DP-control | 12 | 3.3 ± 0.2 | 305 ± 16 | 484,166 ± 87,156 | 2,978 ± 805 | 82.0 ± 2.0 |
| DP-theophylline | 6 | 2.6 ± 0.2 | 506 ± 16 | 193,333 ± 11,155 | 383 ± 20 | 79.1 ± 2.0 |
| DP-control (PD) | 6 | 2.7 ± 0.3 | 467 ± 23 | 213,333 ± 29,401 | 474 ± 89 | 80.0 ± 3.0 |
| DP-control (AD) | 6 | 3.9 ± 0.1 | 143 ± 14* | 755,000 ± 56,730 | 5,480 ± 580* | 83.1 ± 2.0 |

Diabetes-prone (DP) BB rats were fed with water alone (DP-control), or with theophylline (2 mg/ml) added to their drinking water starting at the age of 25 days (DP-Theo). At age 60-75 days pancreases were removed, islets were isolated and mononuclear leukocytes were separated from the islets; their purity was determined by staining with a monoclonal antibody (OX1) to rat leukocytes. Leukocytes obtained from the islets of each rat were studied separately.

Data are means ± SE for the number of rats (n) indicated for the following groups: DR = diabetes-resistant BB rats, DP-control = diabetes-prone BB rats fed with water alone, and DP-Theo = diabetes-prone BB rats fed with theophylline. The DP-control is also shown as broken down into DP-control(PD) = prediabetic diabetes-prone BB rats, and DP-control (AD) acutely-diabetic diabetes-prone BB rats.

Histological examination revealed leukocytes infiltrating the islets in the DR, DP-control, and DP-Theo groups. Islet inflammation (insulitis) was graded from 0 to 4 according to the extent of inflammation: 0 = none, 1 = only peri-islet infiltration, 2 = < 25% islet area infiltrated by leukocytes, 3 = 25-75% islet area infiltrated, and 4 = > 75% islet area infiltrated. The slides were read in a double-blinded fashion.

The asterisks denote statistical significance compared with the DP-Theo group (* = $p < 0.05$) using the Kruskal-Wallis non-parametric ANOVA followed by the Dunn's multiple comparison test (for comprehensive statistical comparisons see text).

# Table 4-2

**Pancreatic insulin content in BB rats**

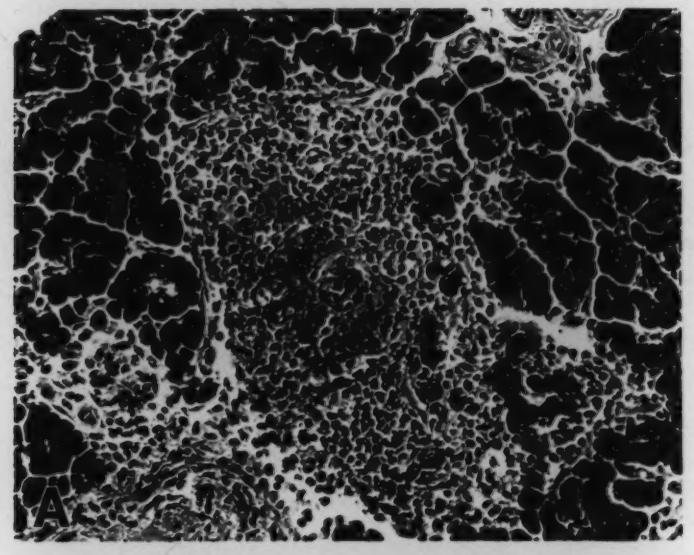
| BB rat group<br>Treatment at 60-75 days | *n* | Blood glucose<br>(mmol/l) | Insulin content<br>(ng/mg pancreas) |
|---|---|---|---|
| DR | 6 | 3.4 ± 0.2 | 48.8 ± 4.0 |
| DP-control | 12 | 10.4 ± 2.2 | 17.8 ± 4.8 |
| DP-theophylline | 6 | 5.2 ± 0.2 | 31.4 ± 4.2 |
| DP-control (PD) | 6 | 3.7 ± 0.1 | 33.3 ± 1.8 |
| DP-control (AD) | 6 | 17.0 ± 2.0 | 2.3 ± 0.6 |

Diabetes-prone (DP) BB rats were fed with water alone (DP-control), or with theophylline (2 mg/ml) added to their drinking water starting at the age of 25 days (DP-Theo). At age 60-75 days, the blood glucose values were obtained and pancreases were removed for determination of insulin content (ng/mg pancreas).

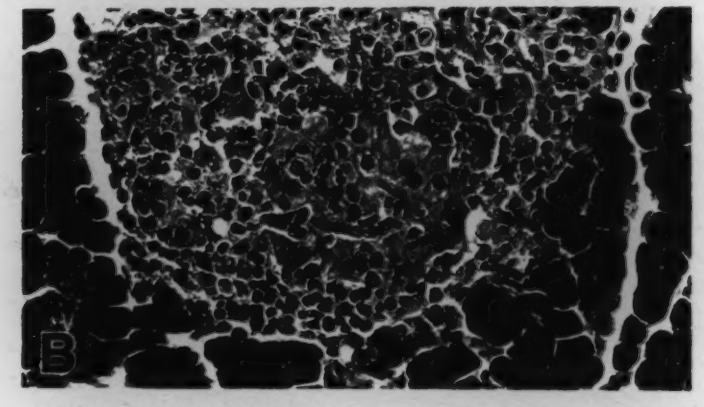
Data are means ± SE for the number of rats (n) indicated for the following groups: DR = diabetes-resistant BB rats, DP-control = diabetes-prone BB rats fed with water alone, and DP-Theo = diabetes-prone BB rats fed with theophylline. The DP-control is also shown as broken down into DP-control(PD) = prediabetic diabetes-prone BB rats, and DP-control (AD) acutely-diabetic diabetes-prone BB rats.

The DP-Theo group was not significantly different from any other group using the Kruskal-Wallis non-parametric ANOVA followed by the Dunn's multiple comparison test (for comprehensive statistical comparisons see text).

## BB Rat Pancreatic Sections (age 60-75 days)




Diabetes-prone (DP), Control




Diabetes-prone (DP), Theophylline treated

50μ

**Fig. 4-3** : Photomicrographs of pancreatic sections from 60- to 75-day-old BB rats. The sections are stained with H&E to reveal the islet-infiltrating mononuclear leukocytes. A : prediabetic DP-BB rat (control) showing a moderately intense pancreatic islet infiltration by mononuclear leukocytes. B: Theo-treated DP-BB rat pancreas with a similar degree of mononuclear leukocyte infiltration as in the pancreas of control DP-BB rats. The degree of islet infiltration by mononuclear leukocytes (insulitis) was scored as described in the legend to Table 4-1.

**3. Isolation of mononuclear leukocytes from pancreatic islets :**

Differences in degrees of insulitis were more completely quantitated in the experiments shown in Table 4-1. The fewest islets were obtained from the acutely diabetic rats (Table 4-21), as expected from the destruction of islets by infiltrating leukocytes in these rats. However, the islets that were isolated from diabetic rats contained the largest numbers of mononuclear leukocytes, followed by those from the DP-control and DP-Theo rats, and the islets of DR-BB rats contained the least (Table 4-1). The purity of mononuclear leukocytes separated from the four groups of rats ranged from a mean of 79% to 83%. No significant differences in the OX1 percentages were found among the groups.

Table 4-1 also reveals that the insulitis scores in the different rat groups, determined by microscopic examination of pancreatic sections, were reflected by proportional differences in numbers of mononuclear leukocytes recovered per isolated islet in the different rat groups.

**4. Cyclophilin steady-state mRNA levels are proportional to numbers of cells submitted to RT-PCR analysis:**

Because samples submitted to RT-PCR analysis contained different numbers of mononuclear leukocytes, it was necessary to demonstrate that mRNA level (PCR product) for cyclophilin, chosen as a house-keeping gene (i.e. constant expression from cell to cell), was proportional to the number of cells in the sample. Fig. 4-4, panel C shows that cyclophilin mRNA expression correlated positively with numbers of islet-infiltrating mononuclear leukocytes (regression coefficient, r= 0.82, $p < 0.0001$). Therefore, cytokine mRNA levels (PCR product) measured in different samples could be compared after normalizing to cyclophilin mRNA levels in the respective samples (as shown in the equation in Chapter 2, section V).

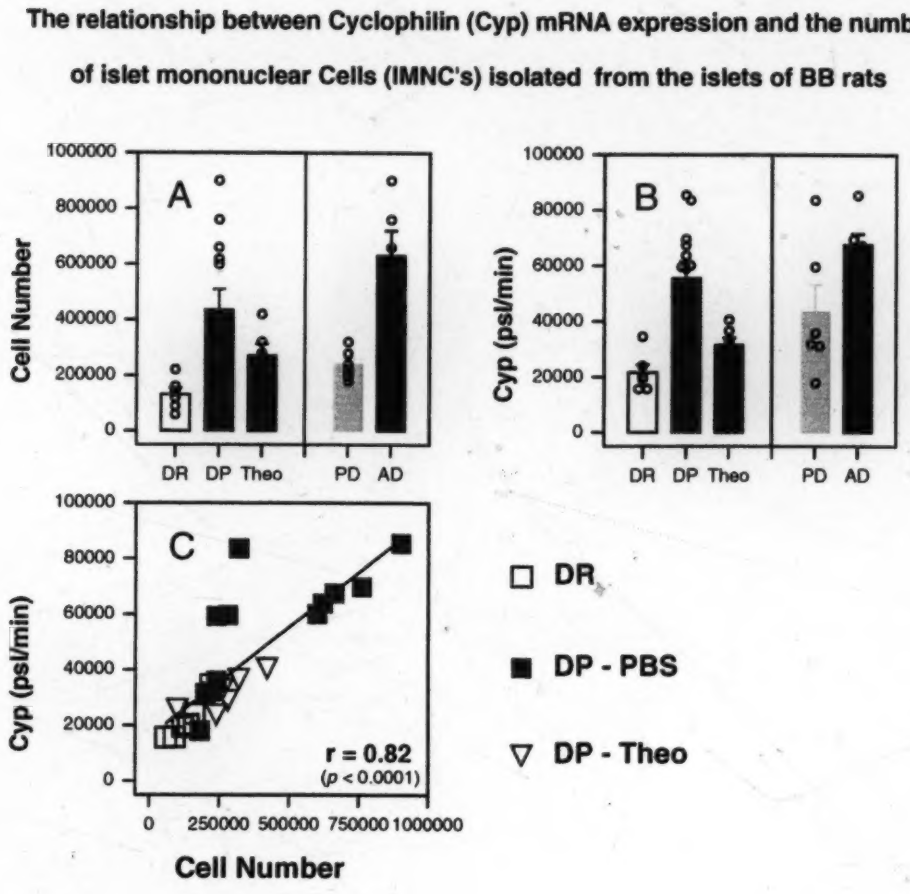



**Fig. 4-4:** The total number of islet mononuclear cells (IMNC's), panel A, and Cyclophilin mRNA levels (Cyp), panel B, and the relationship between them, panel C, in islets of BB rats, ages 60-75 days. DR rats (n=6) received water alone. DP-BB rats received either water alone (DP-PBS, n=12) or water with Theo (DP-Theo, n=6) starting at age 25 days. 50% of the DP-PBS rats were only prediabetic (PD, n=6) at the time of sacrifice, whereas the rest were acutely diabetic (AD). AD rats were studied within 2 days of diabetes onset. Individual animals in each group are shown as open circles (o), and mean values for the groups are indicated by the bars + SE.

**5. Cytokine steady-state mRNA levels in islet mononuclear leukocytes :**

Cytokine steady-state mRNA levels in mononuclear leukocytes isolated from islets of the BB rat groups are shown in Fig. 4-5. IFNγ steady-state mRNA levels were higher in mononuclear leukocytes isolated from islets of the DP-control rats which received only water than in those of the DR group, and the Theo-treated group. The Theo-treated group had levels of IFNγ steady-state mRNA similar to that of the DR group. The steady-state mRNA levels of IL-2, IL-12p40, and IL-10 were also higher in mononuclear leukocytes isolated from islets of the DP-control group than in those of the DR group and the DP-Theo-treated group.

In contrast, the levels of IL-4 and TNFα mRNA were lower in islet mononuclear leukocytes isolated from DP-control group compared with those in the DR group and, Theo treatment of the DP-BB rats was associated with small elevations in the levels of IL-4 and TNFα mRNA in the islet mononuclear leukocytes. However, the differences in the case of all cytokines mentioned did not reach statistical significance.

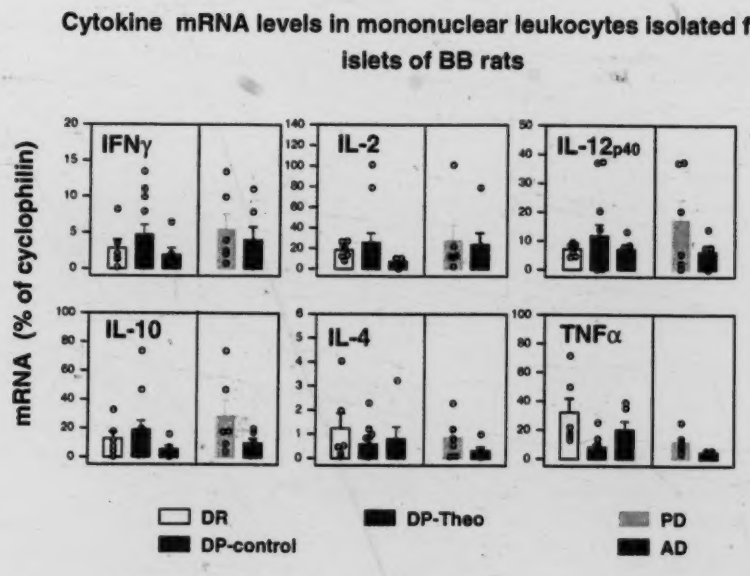



**Fig. 4-5:** Cytokine steady-state mRNA levels in mononuclear leukocytes isolated islets of BB rats, ages 60-75 days. DR rats (n=6, white) received water alone. DP-BB rats received water alone (DP-control, red, n=12) or water with Theophylline (Theo) starting from the age of 25 days (DP-Theo, blue, n=6). 50% of the DP-control rats were only prediabetic (PD, yellow, n=6) at the time of sacrifice, whereas the rest were acutely diabetic (AD, brown, n=6). AD rats were studied within 2 days of diabetes onset. Levels of cytokine mRNA (PCR product) are expressed as a percentage of cyclophilin (Cyp) mRNA. Cytokine mRNA levels (Mean ± SE) are shown for each group of rats, and values for individual animals in each group are shown as open circles (o).

There were also significant correlations between steady-state mRNA levels of IFNγ and IL-2, and IFNγ and IL-10 in the groups of animals involved in the experiment. Fig. 4-6, panel A shows the significant correlation (r= 0.75, $p < 0.0001$) found between the levels of IFNγ and IL-2 steady-state mRNA levels in the BB rats groups, while Fig. 4-6, panel B shows a similar correlation (r= 0.78, $p < 0.0001$) between IFNγ and IL-10 steady-state mRNA levels in the same BB rats groups. Furthermore, Fig. 4-6, panel C shows a similar correlation (r= 0.78, $p < 0.0001$) between IFNγ and IL-12p40 steady-state mRNA levels in the same BB rats groups.

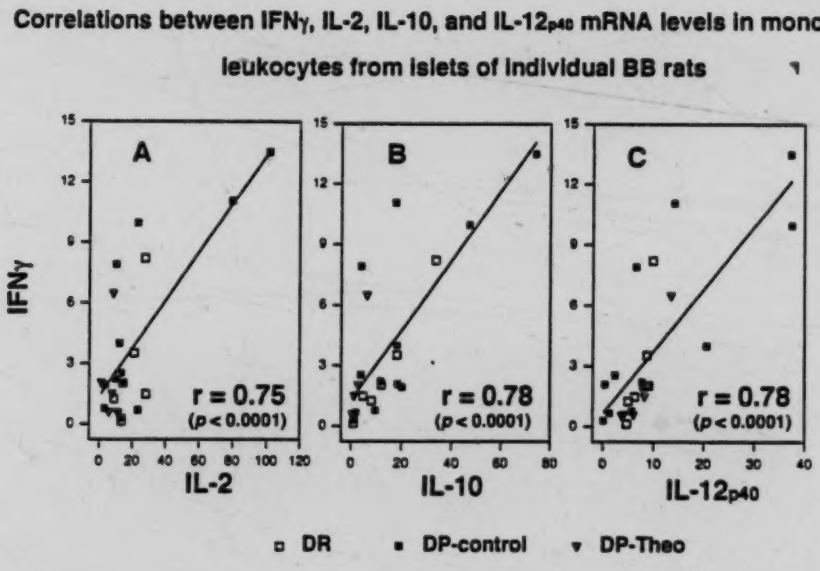



**Fig. 4-6:** Correlations between IFNγ and IL-2 (A), IFNγ and IL-10 (B), and between IFNγ and IL-12p40 (C) mRNA levels in mononuclear leukocytes isolated from islets of individual BB rats: DR (n=6), DP-control (n=12) and DP-Theo (n=6) for animals from the studies described in the legend of Fig. 4-5. The correlation coefficients (r=0.75 for A ($p < 0.0001$), and r=0.78 for B ($p < 0.0001$) and for C, ($p < 0.0001$)) were calculated using SigmaPlot v4.11 (Jandel Scientific).

# III. Discussion

The effect of Theo on diabetes was suspected to be due to the inhibitory effect of Theo on phosphodiesterases which result in the accumulation of cAMP, a function associated with inhibition of mature differentiated lymphocytes (190) and their functions such as cytotoxicity (191). Therefore, I hypothesized that the protective effect of Theo might be mediated through downregulation of type 1 cytokines and consequently their functions, possibly by upregulating type 2 cytokines.

In this present study, oral Theo administration to the DP-BB rats resulted in a moderate reduction of diabetes incidence (approximately 40%), similar to results observed in previous studies (166). The insulitis score and the total number of mononuclear cells isolated from the islets (Table 4-1), indicate that Theo treatment neither prevented the initial invasion of the islets by the infiltrating cells nor reduced the insulitis significantly. However, it appeared to have prevented the transformation of the mild insulitis during prediabetes (Fig. 4-3, panel A, and Table 4-1 DP-control(PD) group) into the aggressive insulitis during acute diabetes (Table 3-2 DP-control(AD) group).

The effect of Theo on diabetes development was moderate. This may be explained by the findings that the effect of the drug on changes in cytokine profiles revealed a trend to decreased type 1

cytokines (IFNγ, IL-2, and IL-12), but this lacked statistical significance. Despite the fact that there were significant correlations among these cytokines and their role in IDDM development is supported by some studies reported in the literature, it is not possible from these Theo treatment studies to conclude that Theo treatment affects the cytokine expression profile due to the lack of statistical significance. A closer examination of the results obtained in case of the cytokine steady-state mRNA levels in the islet mononuclear leukocytes (Fig. 4-5) reveals that the lack of statistical significance among the groups is due to the scatter of the data in the DP-control and also sometimes in the DP-Theo group (e.g. IFNγ). This could be the result of the small number of rats (n=6) used in these experiments. As seen in the CFA administration studies (Table 3-2), statistical significance is obtained when a large number of animals is used for each group. Therefore, by using a larger number of rats for the studies mentioned herein, statistical significance may be obtained. Alternatively, a larger number of rats in each group may provide stronger scatter and consequently lead to no differences among the groups. At any rate, using larger numbers of animals can confirm the statistics either way.

The effect of Theo treatment on the development of IDDM may also be due to other mechanisms. Theo is known to be a general inhibitor of the different isoforms of phosphodiesterases (Type I-V), and different tissues express different isoforms. For example, islet cells were reported to express phosphodiesterases (PDE) III and IV, and inhibition of the former enhanced insulin secretion (195).

Therefore, one of the ways in which Theo moderately protects against the development of IDDM by Theo may be due to the enhancement of insulin secretion.

To test whether Theo offers protection in IDDM development by inhibition of cytotoxicity, future experiments can be done using other PDE inhibitors that are more specific to the PDE IV isoform. This isoform is known to be more important in regulating cAMP levels in lymphocytes.

Pentoxifylline (PTX), an example of a PDE inhibitor which was found to downregulate the biosynthesis of Th1 cytokines while leaving Th2 cytokine production intact (196), maybe more potent in protecting islet β-cells from autoimmune destruction. This drug is also known to inhibit the production of the final effector CTL molecules, granzyme B and perforin (134). Alternatively, Rolipram ((±)-4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone), a specific inhibitor of PDE IV, could be used to study the effect on cytokine expression by macrophages and lymphocytes in diabetes. In fact, both drugs were used in a recent study (197) to prevent diabetes in the NOD mice, and Rolipram was found to be more 100 fold more effective than PTX. However, both PTX and Rolipram were able to reduce the severity of insulitis and incidence of diabetes. Furthermore, these drugs were found to inhibit IL-12 and IFNγ production by macrophages and T-cells respectively. Therefore, it appears that blocking the activity of type IV PDE (by Rolipram) is

sufficient to mediate the effects on type 1 cytokines reported in that study.

In addition to lack of specificity of Theo, the duration of drug administration could have also played an important role. It was observed in human studies that prolonged administration of Theo can results in tachyphylaxis in patients with atopic dermatitis (198). Therefore, it maybe necessary to study the time course effects of Theo to determine if there is a peak of Theo effect on cytokine profile changes which might have been missed by sacrificing the animals only at the time that we did.

# Chapter 5

# The effect of anti-T-cell monoclonal antibody treatments on the development of diabetes and cytokine expression profiles

# I. Introduction

The previous two studies have employed two different approaches that resulted in partial reduction of diabetes incidence in the DP-BB rat. The first approach involved a single injection of the immunostimulant complete Freund's adjuvant (CFA) which delayed and reduced diabetes incidence in DP-BB rats. The second approach dealt with the oral administration of the immunosuppressive drug, Theophylline (Theo) which resulted in significant reduction of diabetes incidence as well. However, only the CFA studies showed that islet inflammation (insulitis) and the development of autoimmune diabetes in the DP-BB rat were associated with the upregulation of type 1 cytokines, especially IFNγ, and that the protective effect of that intervention was associated with downregulation of the type 1 cytokine, IFNγ. However, this downregulation of type 1 cytokines did not appear to be the result of type 2 cytokine upregulation as postulated in the initial hypothesis (Chapter 1, section VI). Thus, neither IL-4 nor IL-10 message was upregulated in the insulitis lesion after treatment of the DP-BB rats with CFA or Theo. Therefore, it appears that CFA downregulated the type 1 cytokine, IFNγ by mechanisms that do not involve changes in type 2 regulatory cytokines, IL-4 and IL-10. Furthermore, the relationship between the two types of cytokines during diabetes development remains obscure since cross-regulation between the two types appeared to be more complex.

Although it was possible to observe an association between IFNγ hyperexpression and IDDM development in the DP-BB rat, the cellular source of IFNγ was not determined in the CFA discussed in Chapter 3. As a result, one cannot conclude that $CD4^+$ Th1 cells alone are responsible for the production of IFNγ as stated in the initial hypothesis. This is because IFNγ could also be secreted by other cells such as $CD8^+$ T-cells (Tc1). Therefore, the experiments described in this chapter were performed in an attempt to address some of these questions.

As mentioned in the introduction, autoimmune diabetes is prevented in diabetes-prone animals treated with monoclonal antibodies directed against T-lymphocytes. In the following experiments, $CD4^+$ and $CD8^+$ T-cells were selectively deleted from DP-BB rats by administration of specific monoclonal antibodies *in vivo* and the effects of these treatments on cytokine expression profiles in the islets and spleen were analyzed to determine which cytokine changes might be associated with prevention of diabetes development by deletion of $CD4^+$ and $CD8^+$ T-cells.

## 1. $CD8^+$ T-cells and autoimmune diabetes:

The first target was the accessory molecule CD8, a glycoprotein present mainly on $T_{cytotoxic/suppressor}$ lymphocytes and NK cells. CD8 functions both as an adhesion molecule and as a TCR co-receptor on $T_{cytotoxic/suppressor}$ lymphocytes, providing supplementary signals to

those generated through the TCR-CD3 complex on MHC class-I-restricted T-cells. The monoclonal antibody (mAb) directed against the CD8 accessory molecule of the BB rat is known as OX8. In one study (199) the injection of tissue culture supernatants containing OX8 (an IgG1 isotype mAb) to DP-BB rats reduced the incidence of diabetes, but not insulitis, in the treated rats. In a subsequent study (53), it was demonstrated that OX8 mAb prevented diabetes development in BB rat by deleting $CD8^+$ T-cells and not deleting $CD8^+$ NK cells because an NK-specific mAb (3.2.3) did not prevent diabetes.

## 2. $CD4^+$ T-cells and autoimmune diabetes:

The first study mentioned above dealing with injection of OX8 mAb to DP-BB rats (199) also examined the effects of injecting other mAbs, most notably W3/25 and OX19. The first mAb, W3/25, is directed against the co-receptor CD4, an analogue to CD8 but present on $T_{helper/inducer}$ cells, and also on macrophages. The second mAb, OX19, is directed against CD5, a pan T-cell adhesion marker. While OX19 treatment was more effective in diabetes prevention and depletion of T-cells, treatment with W3/25 did not result in significant depletion of $CD4^+$ T-cells, or any other subset, and only a small effect on the prevention of diabetes.

Another study (60) reported the use of another group of mAbs directed against the adhesion molecule CD2. This treatment protected against diabetes. CD2 (200) is a 50-kD antigen/signaling and

adhesion molecule expressed on thymocytes, mature T-cells, and NK cells. Treatment of DP-BB rats with a specific anti-CD2 monoclonal antibody (OX34) resulted in significant deletion of $CD5^+/CD8^-$ ($T_{helper/inducer}$ cells), and significant prevention of diabetes development. Therefore, I chose the anti-CD2 mAb OX34 to delete $T_{helper/inducer}$ cells (otherwise known as $CD4^+$ T-cells) from DP-BB rats in the following study.

## 3. Hypothesis:

***By selective deletion of $CD4^+$ T-cells and $CD8^+$ T-cells from DP-BB rats <u>in vivo</u> and analysis of cytokine changes in islets and spleens in relation to prevention of diabetes, the relative contribution of $CD4^+$ and $CD8^+$ T-cells in the development of autoimmune diabetes should be revealed in terms of the cytokines that these T-cell subsets produce and/or use to regulate each other.***

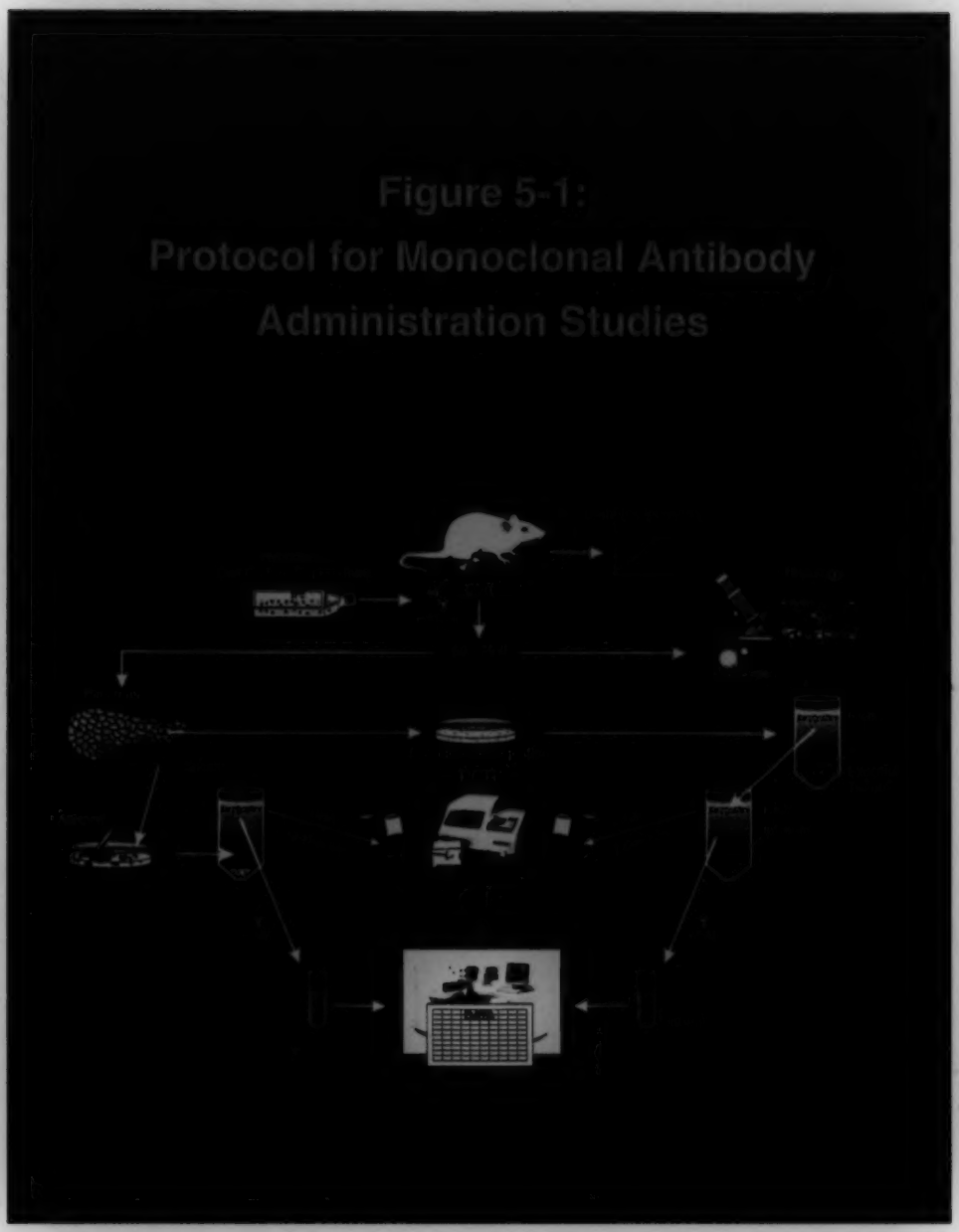
## 4. Protocol:

The experimental protocol is shown in Fig. 5-1. 2 ml of the mouse B-cell hybridoma supernatants containing OX8 (anti-CD8), and OX34 (anti-CD2) anti-rat mAbs were injected i.p. into the DP-BB rats daily starting at the age of 25 days. OX21 was used as a control antibody: it is directed against a human protein, C3bINA, and does

not cross-react with the rat homologue. The hybridoma cultures were grown according to the procedure described in Chapter 2 section II. The approximate amount of the IgG mAb protein in each injection was determined by ELISA assay to be 59.8 μg/ml of OX8, 80 μg/ml of OX21, and 106.5 μg/ml of OX34 ( see Appendix 3 for details). In the first study, the rats were monitored daily for glucosuria, from 50 days of age. Diabetes was defined as the presence of 4+ glucosuria (Tes-tape, Lilly Research Laboratories, CA) and a tail vein plasma glucose ≥ 11 mmol/l measured on a glucometer (Elite, Bayer, CA).

In the second study, anti-CD8-, anti-CD2-treated, and control mAb-treated BB rats were anesthetized with pentobarbital (50 mg/kg of body weight) between the ages of 60-75 days (when incidence of diabetes is approximately 25-50%). Pancreases were excised for histological examination, insulin content assay, mononuclear leukocyte analyses, and cytokine mRNA analyses as described in chapter 2.

Figure 5-1:
Protocol for Monoclonal Antibody
Administration Studies



# II. Results

## 1. Diabetes incidence :

The effect of anti-CD8 and anti-CD2 mAbs treatments (compared to control antibody treatment) on the incidence of diabetes is shown in Fig. 5-2 and Fig. 5-3, respectively. In case of treatment of DP-BB rats with anti-CD8 monoclonal antibody, the incidence of diabetes was delayed. Diabetes started to appear in the experimental group (anti-CD8 mAb) at 98 days of age, while the control group (control mAb) started to develop diabetes at 60 days of age as expected. In addition, the treatment resulted in the overall reduction of diabetes incidence by 67% by the age of 130 days (Fig. 5-2).

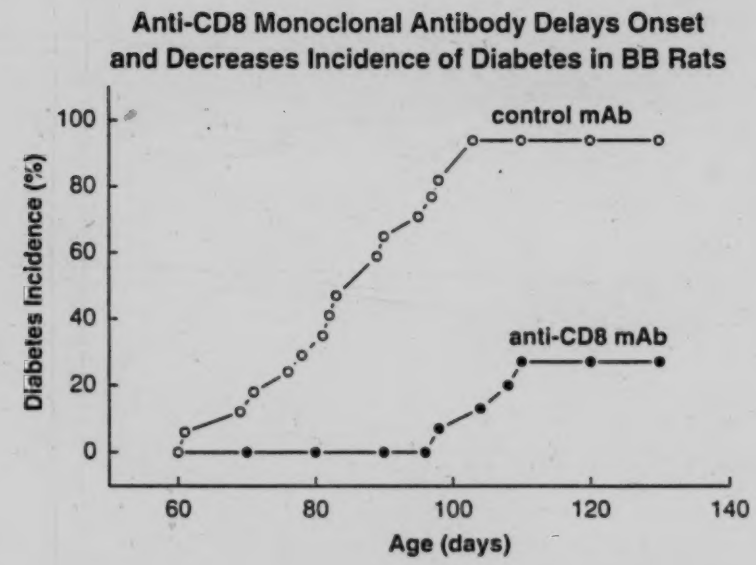



**Fig. 5-2:** BB diabetes-prone (DP) rats were injected i.p. with a control mAb, OX21 (o, n=17) or with anti-CD8 mAb, OX8 (•, n=15), starting at age 25d and continued daily until age 130 days. Diabetes onset was delayed until 98d in anti-CD8-injected DP-BB rats. From age 110 days until 130 days, the incidence of diabetes in the anti-CD8 injected group (26%) was significantly less than in control group (93%), $p < 0.05$ by Chi-squared test, 2x2 tables, df.=1.

Treatment of DP-BB rats with anti-CD2 mAb, on the other hand, has resulted in complete prevention of diabetes until the treatment was discontinued at the age of 120 days. Furthermore, diabetes did not start to develop in this treated group until the age of 150 days (i.e. 30 days after the treatment was discontinued); however, the incidence of diabetes eventually reached the expected 100% without treatment by the age 220 days (Fig. 5-3).

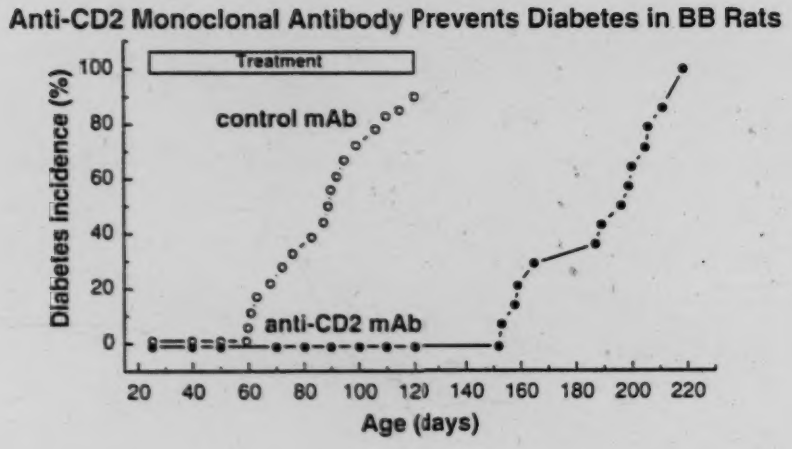



**Fig. 5-3:** BB diabetes-prone (DP) rats were injected i.p. with a control mAb, OX21 (o, n=17), or with anti-CD2 mAb, OX34 (•, n=15), starting at age 25d. Diabetes onset was at 60d of age in the control group, whereas no diabetes developed in the anti-CD2-mAb-treated rats until 33 days after anti-CD2 monoclonal antibody was discontinued (at age 120 days).

## 2. Pancreatic histology, insulitis, and insulin content :

The effect of mAb treatments on the degree of insulitis could be seen from the histological staining of the islet tissues (Fig. 5-4) and the number of mononuclear leukocytes isolated from the islets (Table 5-1). Fig. 5-4, panel A shows an islet of a DR-BB rat, where the insulitis is absent (least number of mononuclear leukocytes/islet, 139 ±11, Table 5-1). In the DP-BB rat group, the infiltrating leukocytes invaded the islets as seen in Fig. 5-4 panels C (prediabetic) and B (acutely-diabetic). This was associated with an increase of the number of mononuclear leukocytes/islet, from 586 ± 60 to 4,033 ± 715 (Table 5-1, DP, control mAb(PD) and control mAb(AD), respectively). Under the protection provided by the mAb treatments, the islets were still infiltrated (Fig. 5-4 D and E), but the number of infiltrating mononuclear leukocytes/islet (Table 5-1) were only significantly decreased from 2,309.5 ± 387.5 in the DP-control mAb to 404 ± 26 with anti-CD2 mAb ($p < 0.05$) treatment, and to 403 ± 37 with anti-CD8 mAb treatment ($p < 0.05$). However, the number of infiltrating mononuclear leukocytes/islet with either treatment was similar to that of the control group before they became diabetic (i.e. 586 ± 60 in the DP, control mAb(PD)). Finally, Table 5-1 shows that the total pancreatic β-cell mass (pancreatic insulin content) in the different groups of BB rats was inversely related to the amount of leukocytic infiltration of the islets in those groups.

In conclusion, monoclonal antibody treatments delayed the onset and significantly decreased the frequency of diabetes development in case of anti-CD8 mAb treatment and completely prevented the development of diabetes in case of anti-CD2 mAb treatment. Neither monoclonal antibody treatment prevented the initial infiltration of islets by mononuclear leukocytes, but significantly decreased the insulitis. However, the total number of mononuclear leukocytes/islet are similar in the DP, anti-CD2 mAb, the DP, anti-CD8 mAb groups and the DP, control mAb(PD) group. Therefore, mAb treatments affect the development of IDDM by preventing the transformation of the islet infiltrate into the aggressive and destructive insulitis (usually seen during acute-diabetes) to the insulin-producing β-cells in the islets by unknown mechanisms.

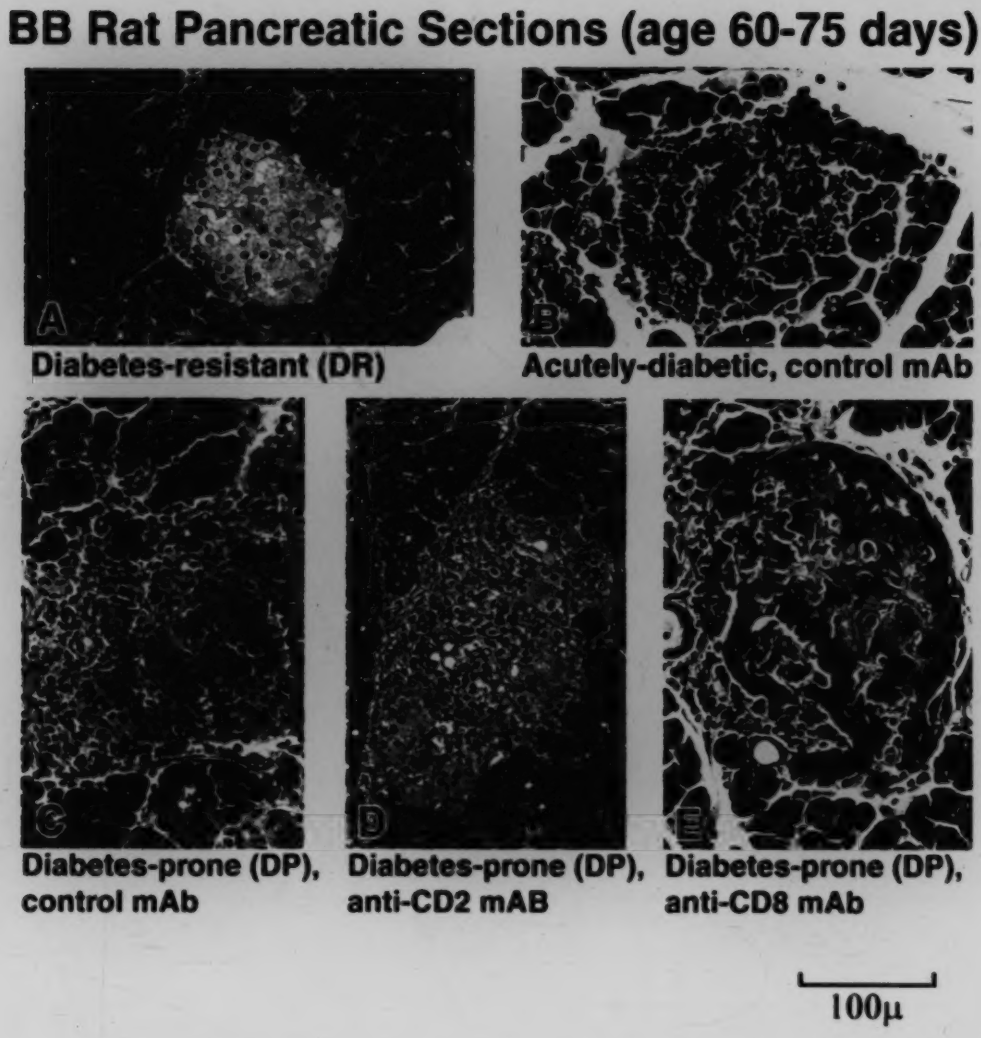



**Fig. 5-4 :** Photomicrographs of pancreatic sections from 60- to 75-day-old BB rats. The sections are stained with H&E to reveal the infiltrating mononuclear leukocytes in spontaneous disease and under the protection provided through the treatment with monoclonal antibody injection. A : DR rat pancreas with intact islet β-cells and few surrounding mononuclear leukocytes. B : acutely diabetic DP-BB rat pancreas with extensive islet infiltration by mononuclear leukocytes and substantial reduction of β-cell mass. C : prediabetic DP-BB rat showing moderate islet infiltration by mononuclear leukocytes and some reduction of β-cell mass. D : anti-CD2-mAb-treated DP-BB rat pancreas with an islet showing less infiltration by mononuclear leukocytes. E : anti-CD8-mAb-treated DP-BB rat pancreas with an islet showing less infiltration by mononuclear leukocytes.

# Table 5-1

**Effects of anti-CD2 and anti-CD8 monoclonal antibody treatments of diabetes-prone BB rats on pancreatic islet infiltration by leukocytes and β-cell destruction**

| BB rat group | Blood glucose (mmol/l) | Insulin content (ng/mg pancreas) | Islets | Mononuclear leukocytes | | |
|---|---|---|---|---|---|---|
| | | | no. per pancreas | Total no. ($x10^{-3}$) | No. per islet | % OX1* |
| DR | 4.1 ± 0.2 | 66.0 ± 3.2* | 565 ± 17* | 77.5 ± 4.0** | 139.0 ± 11.0** | 91 ± 2 |
| DP, control mAb | 9.5 ± 0.8 | 28.0 ± 3.4 | 314 ± 20 | 464.6 ± 30.9 | 2309.5 ± 387.5 | 89 ± 3 |
| DP, anti-CD2 mAb | 4.0 ± 0.1 | 54.6 ± 3.7 | 517 ± 16 | 207.4 ± 11.2* | 404.0 ± 26.0* | 88 ± 3 |
| DP, anti-CD8 mAb | 5.2 ± 0.3 | 54.2 ± 1.3 | 498 ± 24 | 197.1 ± 8.0* | 403.0 ± 37.0* | 90 ± 2 |
| DP, control mAb (PD) | 4.4 ± 0.2 | 43.5 ± 2.1 | 462 ± 24 | 268.3 ± 26.0 | 586.0 ± 60.0 | 88 ± 3 |
| DP, control mAb (AD) | 14.6 ± 1.4 | 12.4 ± 1.3** | 166 ± 16** | 660.8 ± 35.8** | 4033.0 ± 715.0** | 87 ± 2 |

Diabetes-prone (DP) BB rats were injected i.p. daily with 2 ml of hyridoma supernatants containing 160 µg of OX21 (DP, control mAb), 120 µg of OX8 (DP, anti-CD8 mAb), or 213 µg of OX34 (DP, anti-CD2 mAb) beginning at the age of 25 days. At age 60-75 days, the blood glucose values were obtained, pancreases were removed for determination of insulin content (ng/mg pancreas). Pancreases were removed, islets were isolated and mononuclear leukocytes were separated from the islets; their purity was determined by staining with a monoclonal antibody (OX1) to rat leukocytes. Leukocytes obtained from the islets of each rat were studied separately.

Data are means ± SE for the number of rats (n) indicated for the following groups: DR = diabetes-resistant BB rats, DP, control mAb = diabetes-prone BB rats injected with OX21, DP, anti-CD2 mAb = diabetes-prone BB rats injected with OX34, and DP, anti-CD8 mAb = diabetes-prone BB rats injected with OX8. The DP, control mAb is also shown as broken down into DP, control mAb (PD) = prediabetic diabetes-prone BB rats, and DP, control mAb (AD) = acutely-diabetic diabetes-prone BB rats.

The asterisks denote statistical significance compared with the DP, control mAb group (* = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$) using the Kruskal-Wallis non-parametric ANOVA followed by the Dunn's multiple comparison test (for comprehensive statistical comparisons see text).

**3. Effect of anti-T-cell monoclonal antibody treatments on leukocyte subsets in the islets and the spleen:**

Tables 5-2, 5-3, and 5-4 show the effects of monoclonal antibody treatments on the different leukocyte subsets in peripheral blood, spleen, and islets, respectively. Table 5-2 summarizes the effects of monoclonal antibody treatments on leukocytes in peripheral blood. First, the well recognized deficiency of $CD4^+$ and $CD8^+$ T-cells in DP compared to DR rats was observed, along with reciprocal increases in NK cells, macrophages and B-cells in the DP rat. Treatment of the DP-BB rat with anti-CD8 mAb resulted in significant reductions of $CD8^+$ T-cells (85%) and NK cells (96%). This reduction was not accompanied by any changes in $CD4^+$ T-cells, but doubling of macrophages. On the other hand, treatment of the DP-BB rat with anti-CD2 mAb resulted in significant reduction, or almost complete depletion of $CD4^+$ T-cells (87%) and NK cells (69%) but no change in $CD8^+$ T-cells. Macrophages were also doubled by anti-CD2 mAb treatment. No changes were seen in B-cell populations.

# Table 5-2

**Leukocyte subsets in peripheral blood of diabetes-prone BB rats treated with anti-CD2 and anti-CD8 monoclonal antibodies[a]**

| BB rat group | Tissue | Mononuclear leukocyte subset (% of total leukocytes)[b] | | | | |
|---|---|---|---|---|---|---|
| | | CD4 (W3/25+) | CD8 (OX8+) | NK (3.2.3+) | Mϕ (OX42+) | B (OX12+) |
| DR | Blood | 29.8 ± 2.7** | 27.5 ± 1.0** | 1.2 ± 0.1* | 3.8 ± 0.4 | 35.0 ± 2.5** |
| DP, control mAb | Blood | 6.1 ± 0.4 | 8.4 ± 0.5 | 5.2 ± 0.4 | 9.4 ± 1.6 | 58.0 ± 4.6 |
| DP, anti-CD2 mAb | Blood | 0.8 ± 0.1* | 10.9 ± 0.5 | 1.6 ± 0.2* | 18.5 ± 1.0* | 59.0 ± 4.5 |
| DP, anti-CD8 mAb | Blood | 5.7 ± 0.7 | 1.3 ± 0.2* | 0.2 ± 0.1** | 18.4 ± 0.8* | 82.0 ± 2.5 |

[a] Diabetes-prone (DP) BB rats received a daily intraperitoneal injection of anti-CD2 mAb (OX34), anti-CD8 mAb (OX-8), and control mAb (OX-21) beginning at age 25 days. DR are diabetes-resistant BB rats.

[b] Leukocytes in peripheral blood were collected at age 60-75 days, then stained with mAbs to the different leukocyte subsets indicated and analyzed by FACS. Mean ± SE values are shown for eight rats from each group.

*$p<0.05$, **$p<0.01$, ***$p<0.001$ vs DP, control mAb

Table 5-3 summarizes the effects of monoclonal antibody treatments on the leukocytes in the spleen. First, as had been seen in peripheral blood (Table 5-2), $CD4^+$ and $CD8^+$ T-cells were significantly reduced in spleens of DP compared to DR rats, whereas NK cells, macrophages and B-cells were significantly increased in the DP rats. Treatment of the DP-BB rats with anti-CD8 mAb resulted in significant reductions of $CD8^+$ T-cells (61%) and NK cells (70%). This reduction was accompanied by a significant increase of $CD4^+$ T-cells. On the other hand, treatment of the DP-BB rats with anti-CD2 mAb resulted in significant reduction of $CD4^+$ T-cells (54%) and a small increase in $CD8^+$ T-cells. No significant changes were seen in macrophage and B-cell populations after treatment with either anti-CD2 and anti-CD8 mAb.

# Table 5-3

**Leukocyte subsets in spleens of diabetes-prone BB rats treated with anti-CD2 and anti-CD8 monoclonal antibodies[a]**

| BB rat group | Tissue | Mononuclear leukocyte subset (% of total leukocytes)[b] | | | | |
|---|---|---|---|---|---|---|
| | | CD4 (W3/25$^+$) | CD8 (OX8$^+$) | NK (3.2.3$^+$) | Mφ (OX42$^+$) | B (OX12$^+$) |
| DR | Spleen | 40.7 ± 1.5** | 27.8 ± 1.0** | 2.4 ± 0.2*** | 5.4 ± 0.4* | 24.4 ± 2.8*** |
| DP, control mAb | Spleen | 12.0 ± 0.3 | 9.8 ± 1.2 | 9.4 ± 0.8 | 15.8 ± 0.6 | 52.6 ± 2.7 |
| DP, anti-CD2 mAb | Spleen | 5.5 ± 0.5* | 15.5 ± 0.9 | 8.3 ± 0.8 | 19.8 ± 2.0 | 48.1 ± 1.9 |
| DP, anti-CD8 mAb | Spleen | 22.9 ± 0.8* | 3.8 ± 0.4* | 2.8 ± 0.2** | 18.4 ± 2.5 | 51.6 ± 1.7 |

[a] Diabetes-prone (DP) BB rats received a daily intraperitoneal injection of anti-CD2 mAb (OX34), anti-CD8 mAb (OX-8), and control mAb (OX-21) beginning at age 25 days. DR are diabetes-resistant BB rats.

[b] Leukocytes in spleens were collected at age 60-75 days, then stained with mAbs to the different leukocyte subsets indicated and analyzed by FACS. Mean ± SE values are shown for eight rats from each group.

*$p$<0.05, **$p$<0.01, ***$p$<0.001 vs DP, control mAb

Table 5-4 summarizes the effects of mAb treatments on the leukocytes in the islets. First, as had been seen in peripheral blood (Table 5-2) and spleen (Table 5-3), $CD4^+$ and $CD8^+$ T-cells were significantly reduced in islets of DP compared to DR rats, whereas NK cells, macrophages and B-cells were significantly increased in the DP rats. Treatment of the DP-BB rats with anti-CD8 mAb resulted in a significant reduction in the $CD8^+$ T-cells in the islets (66%), and NK cells (58%), and a small increase in $CD4^+$ T-cells in the islets. On the other hand, treatment of the DP-BB rats with anti-CD2 mAb resulted in almost complete depletion (80%) of $CD4^+$ T-cells in the islets. This was accompanied by a significant increase in $CD8^+$ T-cells. No significant changes were seen in macrophages or B-cell populations after treatment with either anti-CD2 or anti-CD8 mAb.

# Table 5-4

**Leukocyte subsets in islets of diabetes-prone BB rats treated with anti-CD2 and anti-CD8 monoclonal antibodies[a]**

| BB rat group | Tissue | Mononuclear leukocyte subset (% of total leukocytes)[b] | | | | |
|---|---|---|---|---|---|---|
| | | CD4 (W3/25+) | CD8 (OX8+) | NK (3.2.3+) | MΦ (OX42+) | B (OX12+) |
| DR | Islet | 39.5 ± 4.0** | 32.9 ± 4.0* | 1.1 ± 0.1*** | 3.3 ± 0.6*** | 29.4 ± 2.1* |
| DP, control mAb | Islet | 13.1 ± 1.0 | 11.3 ± 1.9 | 7.3 ± 0.8 | 17.0 ± 1.1 | 52.1 ± 3.8 |
| DP, anti-CD2 mAb | Islet | 2.6 ± 0.6* | 24.3 ± 2.6* | 4.6 ± 0.8 | 11.1 ± 0.7 | 57.6 ± 3.6 |
| DP, anti-CD8 mAb | Islet | 17.8 ± 1.0 | 3.8 ± 0.5* | 3.1 ± 0.4* | 15.0 ± 1.3 | 55.5 ± 5.1 |

[a] Diabetes-prone (DP) BB rats received a daily intraperitoneal injection of anti-CD2 mAb (OX34), anti-CD8 mAb (OX-8), and control mAb (OX-21) beginning at age 25 days. DR are diabetes-resistant BB rats.

[b] Leukocytes in islets were collected at age 60-75 days, then stained with mAbs to the different leukocyte subsets indicated and analyzed by FACS. Mean ± SE values are shown for eight rats from each group.

*$p<0.05$, **$p<0.01$, ***$p<0.001$ vs DP, control mAb

**4. Cyclophilin steady-state mRNA levels are proportional to numbers of cells submitted to RT-PCR analysis:**

Fig. 5-5 and Fig. 5-6 show the relationship between the number of total mononuclear leukocytes extracted for RNA and submitted to RT-PCR analysis from the insulitis lesion and spleen, respectively, and the cyclophilin (Cyp) message which, in theory, should be constant from cell to cell, in the four groups of rats used in this study, i.e., the level of expression of Cyp should reflect the number of cells in the samples. Fig. 5-5, panel C shows that Cyp mRNA expression correlates positively with the numbers of islet-infiltrating mononuclear leukocytes (regression coefficient, $r = 0.74$, $p < 0.0001$)

**The relationship between Cyclophilin (Cyp) mRNA expression and the number of islet mononuclear Cells (IMNC's) isolated from the islets of BB rats**

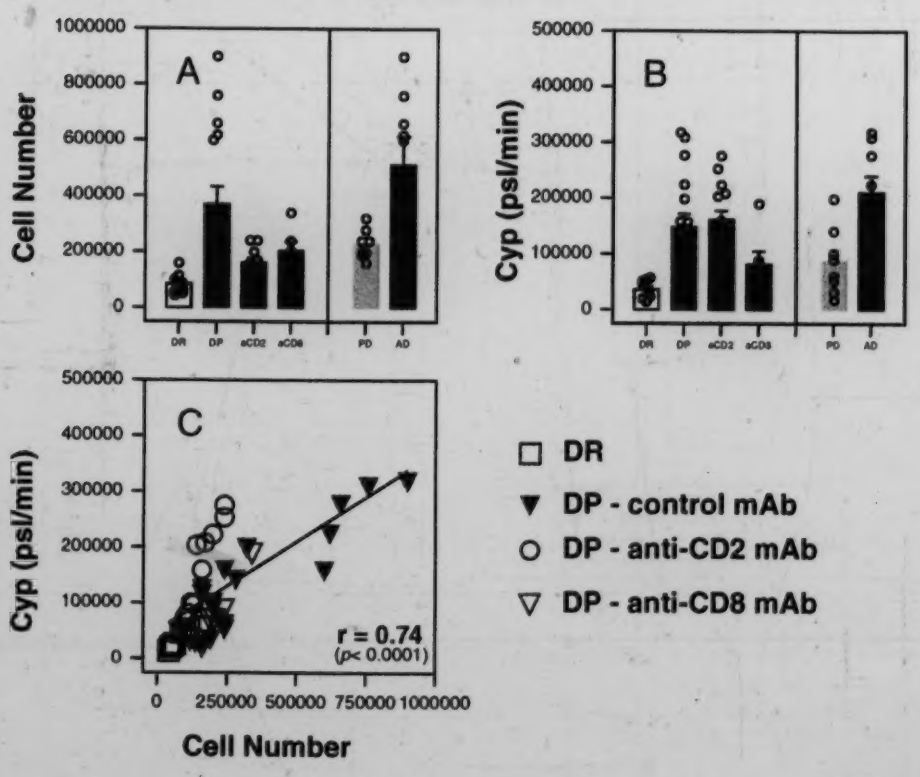



**Fig. 5-5:** The total number of islet mononuclear cells (IMNC's), panel A, and Cyclophilin mRNA levels (Cyp), panel B, and the relationship between them, panel C, in islets of BB rats, ages 60-75 days. DR rats (n=8, white) are diabetes-resistant BB rats. DP-BB rats were injected daily starting at age 25 days with a control mAb, OX21 (DP-control, n=16, red) or either anti-CD2 (DP-anti-CD2, n=10, green) or with anti-CD8 (DP-anti-CD8, n=10, blue) mAbs. 50% of the DP-control rats were prediabetic (PD, n=8, yellow) at the time of sacrifice, whereas the rest were acutely diabetic (AD, n=8, darker red). AD rats were studied within 2 days of diabetes onset. Individual animals in each group are shown as open circles (o), and mean values for the groups are indicated by the bars + SE.

Similarly, Fig. 5-6, panels C, shows that Cyp message correlates positively (regression coefficient, r = 0.78, $p < 0.0001$) with the number of splenocytes isolated from these animals.

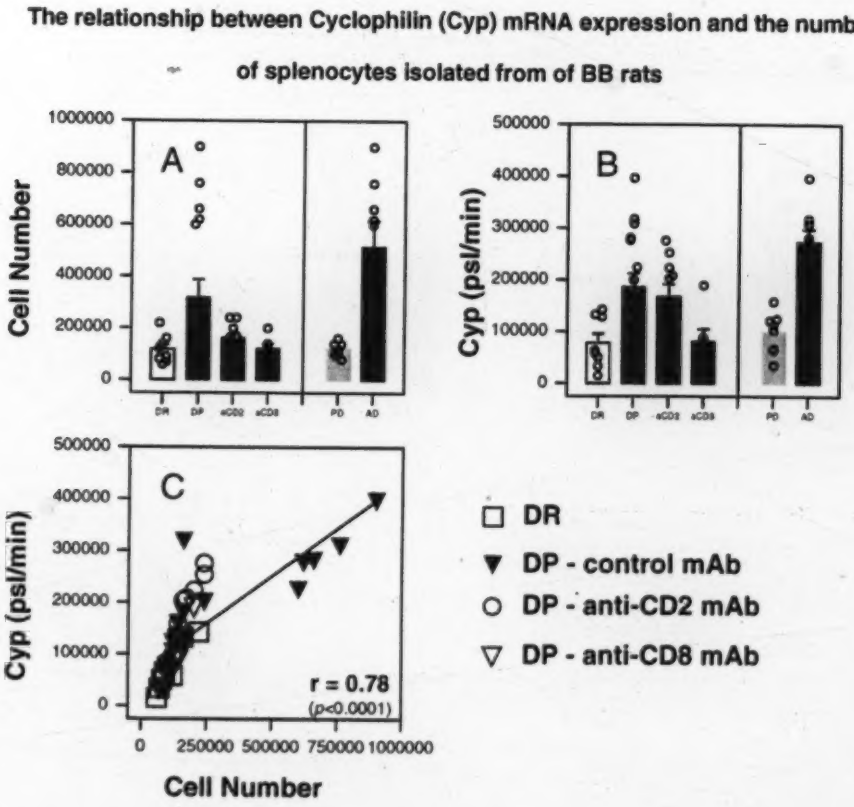



**Fig. 5-6:** The total number of splenocytes (Sp), panel A, and Cyclophilin mRNA levels (Cyp), panel B, and the relationship between them, panel C, in islets of BB rats, ages 60-75 days. DR rats (n=8, white) are diabetes-resistant BB rats. DP-BB rats were injected daily starting at age 25 days with a control mAb, OX21 (DP-control, n=16, red) or either anti-CD2 (DP-anti-CD2, n=10, green) or with anti-CD8 (DP-anti-CD8, n=10, blue) mAbs. 50% of the DP-control rats were prediabetic (PD, n=8, yellow) at the time of sacrifice, whereas the rest were acutely diabetic (AD, n=8, darker red). AD rats were studied within 2 days of diabetes onset. Individual animals in each group are shown as open circles (o), and mean values for the groups are indicated by the bars + SE.

**5. Cytokine steady-state mRNA levels in islet mononuclear leukocytes :**

Cytokine steady-state mRNA levels in mononuclear leukocytes isolated from islets of the BB rat groups are shown in Fig. 5-7. IFNγ steady-state mRNA level was significantly upregulated in islet mononuclear leukocytes of the DP-BB rat compared with the DR-BB group ($p < 0.001$), as had been seen in the CFA studies. Treatment of the DP-BB rats with either anti-CD2 ($p < 0.001$) or anti-CD8 ($p < 0.01$) mAb resulted in significant downregulation of IFNγ steady-state mRNA level. IL-2 steady-state mRNA level was also downregulated by treating DP-BB rats with anti-CD2 mAb ($p < 0.01$) but not anti-CD8 mAb, while IL-12p40 steady-state mRNA level was downregulated by treatment with anti-CD8 mAb ($p < 0.01$) but not anti-CD2 mAb. In addition, treatments with either anti-CD2 or anti-CD8 mAb resulted in decreased IL-10 steady-state mRNA level in DP-BB rats, however, the decreases were not statistically significant ($p = 0.06$).

IL-4 mRNA expression was downregulated in the DP-BB rat compared with the DR-BB rat (albeit not significantly). Also, IL-4 steady-state mRNA level was not affected with anti-CD2 mAb treatment but was significantly upregulated with anti-CD8 mAb treatment ($p < 0.05$). TNFα steady-state mRNA level resembled that of IL-4; however, the upregulation of TNFα steady-state mRNA level with anti-CD8 mAb treatment lacked statistical significance. There were no significant changes in the levels of IL-1β or TGFβ1 messages

among the different BB rat groups. No significant differences were seen in the levels of any cytokine steady-state mRNA level between prediabetic (PD) and acutely diabetic (AD) subgroups of the DP-control rats.

In conclusion, both anti-CD2 and anti-CD8 monoclonal antibody treatments of the DP-BB rats resulted in significant downregulation of the type 1 cytokine, IFNγ in the insulitis lesion, whereas the effects on the other Type I cytokines IL-2 and IL-$12_{p40}$ differed depending on the particular mAb. Regarding the type 2 cytokines (IL-4 and IL-10) IL-4 steady-state mRNA level was upregulated by anti-CD8 but not anti-CD2 mAb treatment, and IL-10 steady-state mRNA level was downregulated (albeit not significantly) by both anti-CD2 and anti-CD8 mAb treatments. These findings reveal that : i) <u>both</u> $CD4^+$ and $CD8^+$ T-cells must be present for IFNγ expression in the insulitis lesion; ii) abrogation of IFNγ expression by deletion of either $CD4^+$ or $CD8^+$ T-cells from the insulitis lesion may be <u>sufficient</u> to prevent β-cell destruction and diabetes development, despite the expression of one or another "pro-inflammatory" cytokine (IL-2, IL-$12_{p40}$, IL-1β or TNFα) that persisted when either $CD4^+$ or $CD8^+$ T-cells were deleted; and iii) upregulation of type 2 cytokines, IL-4 and IL-10, is <u>not</u> necessary for downregulation of IFNγ steady-state mRNA level and prevention of diabetes development.

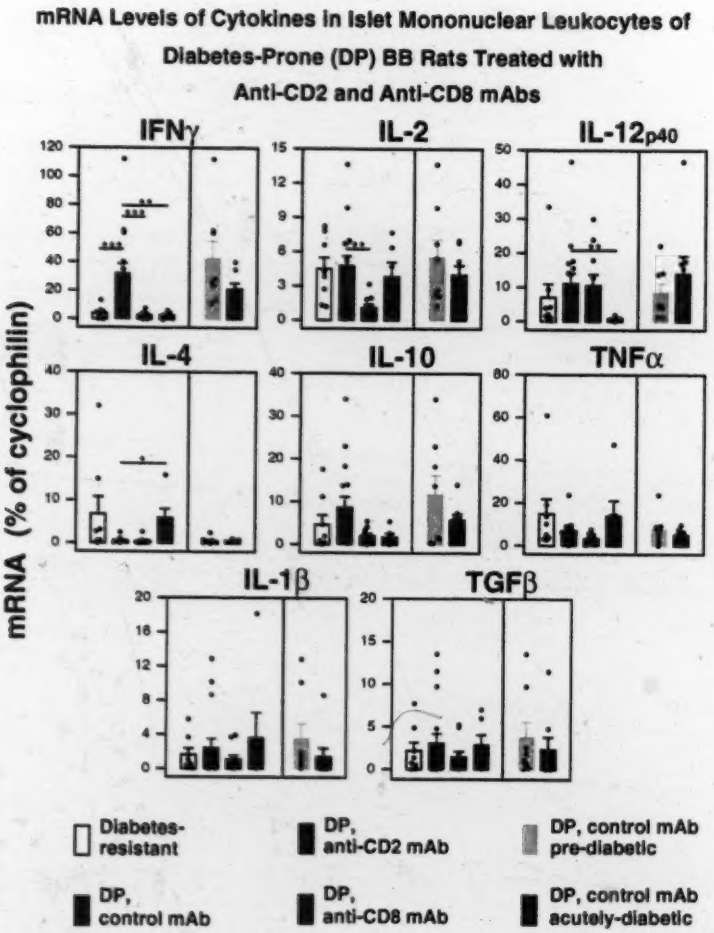



**Fig. 5-7:** Cytokine mRNA levels in mononuclear leukocytes from isolated islets of BB rats, ages 60-75 days. DR-BB rats (white, n=8) are diabetes-resistant BB rats. DP-BB rats were injected daily, starting at the age of 25d, with a control mAb, OX21 (red, n=16), an anti-CD2 mAb, OX34(green, n=10), or an anti-CD8 mAb, OX8(blue, n=6). The control group was broken-down into pre-diabetic rats (yellow, n=8), and acutely-diabetic rats (darker red, n=8) which were studied within 2 days of diabetes onset. Levels of cytokine mRNA (PCR product) are expressed as a percentage of cyclophilin (Cyp). Individual animals in each group are shown as open circles (o). Statistical significance was determined by a non-parametric ANOVA test (Kruskal-Wallis). * $p < 0.05$, ** $p < 0.01$ ***$p < 0.001$ vs. DP, control mAb-treated group.

**6. Cytokine steady-state mRNA levels in the spleen :**

In the spleen, the only significant effects of the mAb treatments were seen in case of IL-4 and TNFα messages (Fig. 5-8). IL-4 steady-state mRNA level was significantly upregulated in DP-BB rats with either anti-CD2 ($p < 0.05$) or anti-CD8 ($p < 0.01$) mAb treatments. Similarly, TNFα steady-state mRNA level was significantly upregulated with anti-CD2 ($p < 0.001$) and with anti-CD8 ($p < 0.01$) mAb treatments. The other cytokines examined in this study were not significantly affected by either treatment (Fig. 5-8). In addition, no significant differences were seen in the levels of any cytokine steady-state mRNA level between prediabetic (PD) and acutely diabetic (AD) subgroups of the DP-control rats (Fig. 5-8).

**In conclusion, both anti-CD2 and anti-CD8 monoclonal antibody treatments of DP-BB rats, which protected against the development of diabetes, resulted in the increased IL-4 and TNFα steady-state mRNA levels in the spleen. This suggests that deletion of either $CD4^+$ and $CD8^+$ T-cells from diabetes-prone BB rats may prevent diabetes development by mechanisms involving systemic (splenic) upregulation of IL-4 and TNFα production. This conclusion is concordant with reports that systemic administrations of IL-4 and TNFα can decrease diabetes incidence in diabetes-prone NOD mice and/or BB rats.**

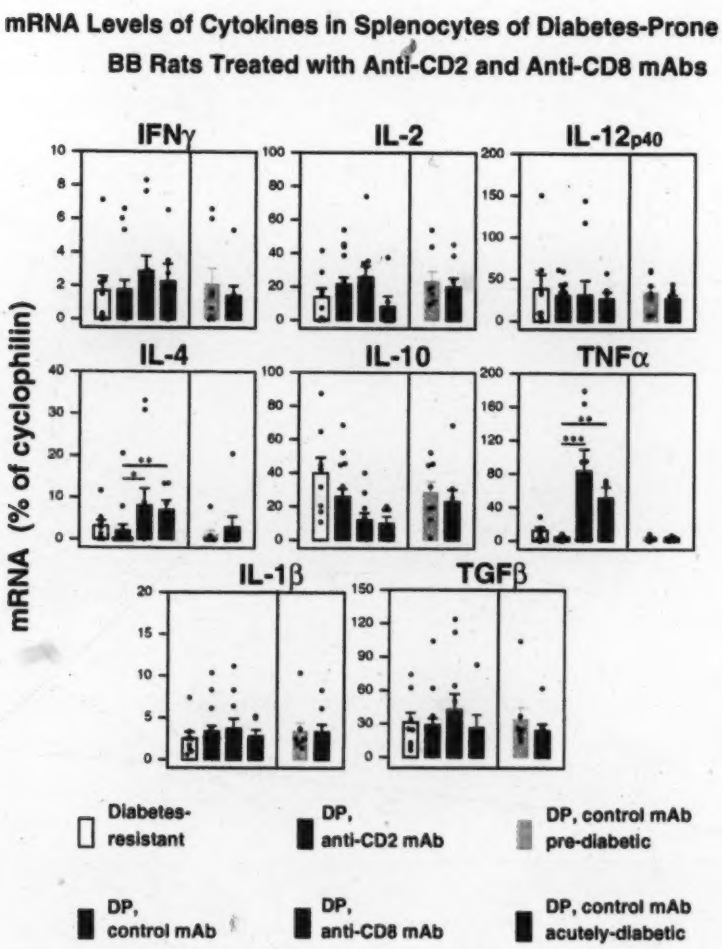



**Fig. 5-8:** Cytokine mRNA levels in splenocytes isolated from BB rats, ages 60-75 days. DR-BB rats (white, n=8) are diabetes-resistant BB rats. DP-BB rats were injected daily, starting at the age of 25d, with a control mAb, OX21 (red, n=16), an anti-CD2 mAb, OX34(green, n=10), or an anti-CD8 mAb, OX8(blue, n=6). The control group was broken-down into pre-diabetic rats (yellow, n=8), and acutely-diabetic rats (darker red, n=8) which were studied within 2 days of diabetes onset. Levels of cytokine mRNA (PCR product) are expressed as a percentage of cyclophilin (Cyp). Individual animals in each group are shown as open circles (o). Statistical significance was determined by a non-parametric ANOVA test (Kruskal-Wallis). * $p < 0.05$, ** $p < 0.01$ ***$p < 0.001$ vs. DP, control mAb-treated group.

**7. Significant correlations among cytokines in the islets:**

The correlations between type 1 cytokines, i.e. between IFNγ and IL-2, and between IFNγ and IL-12p40, in the insulitis lesion of the four groups of animals studied were found to be not significant due to the selective effect of anti-CD2 mAb treatment on IL-2 expression and that of anti-CD8 mAb treatment on IL-12p40. However, significant correlations between IFNγ and IL-10 and between TNFα and IL-4 were found. Fig. 5-9, panel A shows the significant positive correlation found between IFNγ and IL-10 steady-state mRNA levels in the islets ($r = 0.83$, $p < 0.0001$), whereas no correlation was found between these cytokines in the spleens of these animals (Fig. 5-9, panel B). Fig. 5-10, panel A shows the significant positive correlation between TNFα and IL-4 steady-state mRNA levels in the islets ($r = 0.85$, $p < 0.0001$), whereas only a moderate correlation was found between these cytokines in the spleens of these animals ($r = 0.33$, $p < 0.05$) (Fig. 5-10, panel B).

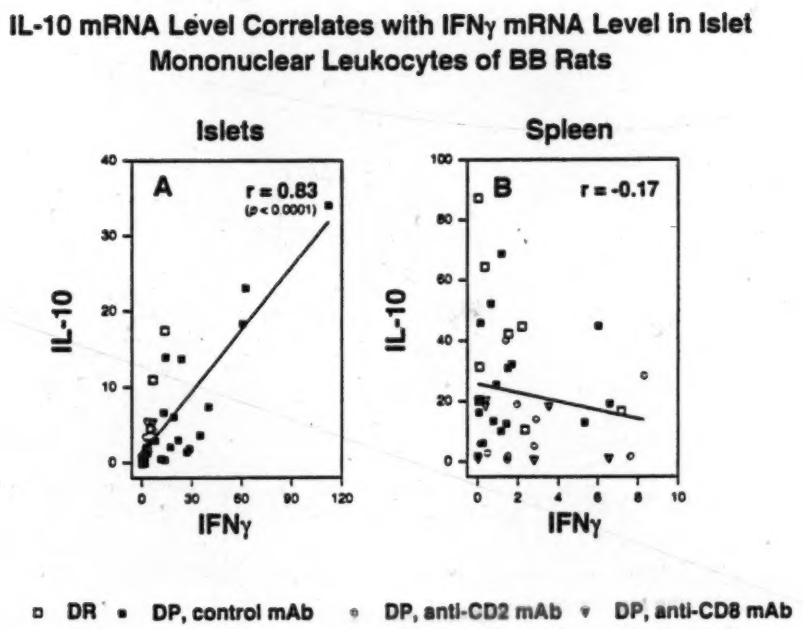



**Fig. 5-9:** Correlations between IFNγ and IL-10, in mononuclear leukocytes isolated from the islets (A) and the spleens (B) of individual BB rats: DR (n=8), DP-control mAb (n=16), DP-anti-CD2 mAb (n=10), and DP-anti-CD8 mAb (n=6 treated as described in the legend of Fig. 5-7. The correlation coefficients (r= 0.83 for A, and r= -0.17 for B) were calculated using SigmaPlot v4.11 (Jandel Scientific).

**TNFα mRNA Level Correlates with IL-4 mRNA Level in Islet Mononuclear Leukocytes of BB Rats**

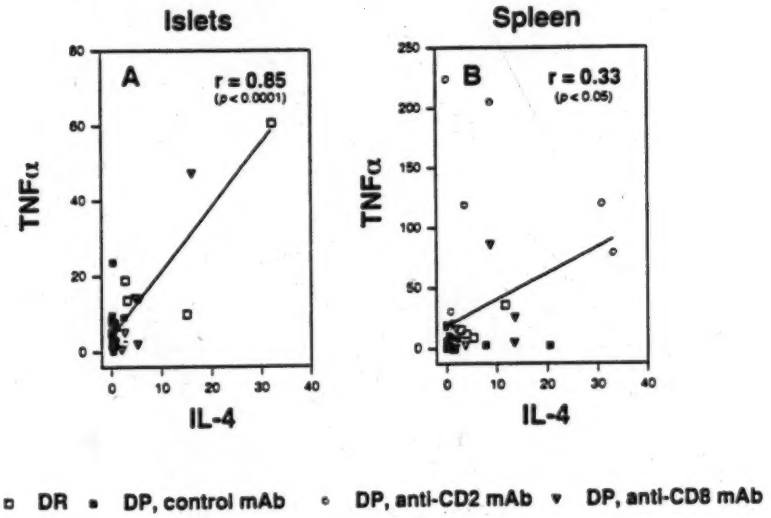



**Fig. 5-10:** Correlations between TNFα and IL-4, in mononuclear leukocytes isolated from the islets (A) and the spleens (B) of individual BB rats: DR (n=8), DP-PBS (n=16), DP-anti-CD2 mAb (n=10), and DP-anti-CD8 mAb (n=6) treated as described in the legend of Fig. 5-7. The correlation coefficients (r= 0.85 for A, and r= 0.33 for B) were calculated using SigmaPlot v4.11 (Jandel Scientific).

In conclusion, the correlations suggest that IL-10 steady-state mRNA level in the islets is coordinately and not reciprocally regulated with IFNγ steady-state mRNA level, i.e. that IL-10 may be pro-inflammatory (and not suppressive) in the islets along with IFNγ. Also, the correlations suggest that the TNFα steady-state mRNA level in the islets is coordinately regulated with IL-4 steady-state mRNA level, i.e. that TNFα may be regulatory rather than pro-inflammatory in the islets along with IL-4.

# III. Discussion

The current studies confirm the findings of previous studies investigating the effect of anti-CD8 and anti-CD2 mAb injections on the development of diabetes in the BB rat. Thus, anti-CD8 mAb (OX8) significantly decreased diabetes incidence in the DP-BB rats and this reduction is primarily due to deletion of $CD8^+$ T-cells and not NK cells (199), (53). Anti-CD2 mAb (OX34) treatment was more effective in the prevention of diabetes and it was primarily directed toward depleting $CD4^+$ T-cells (60).

In previous studies, the hybridoma supernatants were injected intraperitoneally in DP-BB rats without knowing the actual amount of monoclonal antibody being injected into the animals. In my studies, we determined the amount of the particular immunoglobulin (Ig) isotype (e.g. IgG1 or IgG2a) of the mAb as well as the amount of total protein in the hybridoma supernatants injected into the DP-BB rats. The amount of IgG in the supernatants was equal to 5-10% of total protein (Appendix 3). Also, the 1% FBS present in the hybridoma supernatants contributed only another 10% of the total protein in the supernatants. Therefore, there was approximately 80% of unknown proteins injected into the animals along with the specific mAbs. This was unexpected since these were supposed to be non-secretory hybridoma cell lines. Therefore, it is possible that all this unidentified protein resulted from the cellular debris due to cell death during growing the cultures.

Nevertheless, it appears that the effects of the hybridoma supernatant injections on diabetes were due to the specific monoclonal antibody in the injections since the control treatment with hybridoma supernatants containing IgG1 isotype mAb directed against a human protein did not have any effect on the development of diabetes. Furthermore, FACS analysis showed that the treatments were effective in deleting the desired T-cell subsets.

With respect to the effect on diabetes, similar results to what was previously reported in the literature were obtained in these current studies. While anti-CD8 mAb treatment delayed (38 days) and moderately reduced (67%) the incidence of diabetes in the treated rats (Fig. 5-2), anti-CD2 mAb treatment resulted in complete prevention (100%) of diabetes in the treated rats during the time of treatment and for 33 days after the treatment was discontinued (Fig. 5-3). This indicates that prevention of diabetes requires continued mAb administration and deletion of $CD4^+$ T-cells is more effective in diabetes prevention than deletion of $CD8^+$ T-cells suggesting a more important role for $CD4^+$ T-cells in diabetes development.

FACS analysis, which were performed on three compartments of the body [the blood (Table 5-2), the spleen (Table 5-3), and the islets (Table 5-4)] showed that the mAb treatments were effective in deleting the desired subsets, most importantly in the islets. There was a selective and substantial reduction (66%) of $CD8^+$ leukocytes (namely $CD8^+$ T-cells and NK cells) by the anti-CD8 mAb treatment,

and an even greater and selective reduction (80%) of $CD4^+$ T-cells by the anti-CD2 mAb treatment. These results indicated that the protection against diabetes development was directly related to the deletion of $CD4^+$ T-cells (by anti-CD2-mAb). In the case of anti-CD8-mAb, it can be assumed that diabetes was prevented as a result of $CD8^+$ T-cell deletion and not from $CD8^+$ NK cell deletion, because it has previously been reported that deletion of NK cells alone did not prevent diabetes in the DP-BB rats (53).

The effectiveness of anti-CD2 mAb treatment on diabetes incidence could be attributable to its somewhat greater ability to remove the $CD4^+$ T-cells than that of anti-CD8 mAb to remove $CD8^+$ T-cells. Nevertheless, $CD8^+$ T-cells without $CD4^+$ T-cells did not appear to have much impact on diabetes development, despite being significantly increased in the islets when $CD4^+$ T-cells were deleted (Table 5-4). On the other hand, the "break-through" of diabetes development in DP-BB rats treated with anti-CD8 mAb may be related to the small increase of $CD4^+$ T-cells in the islets in the $CD8^+$-T-cell-depleted animals (Table 5-4).

Whereas FACS analysis of leukocyte subsets after mAb treatments showed the relationships between the cellular subsets in question ($CD4^+$ and $CD8^+$ T-cells) and their possible roles in the development of diabetes, interactions between leukocyte subsets, as well as between leukocytes and the target β-cells, may be understood better by analyzing cytokines, i.e. the products of leukocytes which affect their functions. Table 5-5 contains a

summary of the effects of mAb treatments on the changes in cytokine gene expression in the islets and spleens of the DP-BB rats.

## Table 5-5

**Correlations of cytokine mRNA levels in islets and spleens with protection from diabetes in BB rats treated with anti-CD2 and anti-CD8 monoclonal antibodies**

| | IFNγ | IL-2 | IL-12 | TNFα | IL-4 | IL-10 |
|---|---|---|---|---|---|---|
| **Islets** | | | | | | |
| anti-CD2 | –*** | –** | 0 | 0 | 0 | – |
| anti-CD8 | –** | 0 | –** | + | +* | – |
| **Spleens** | | | | | | |
| anti-CD2 | 0 | 0 | 0 | +*** | +* | – |
| anti-CD8 | 0 | – | 0 | +** | +** | – |

cytokine mRNA level decreased (–), increased (+), not changed (0)
*$p < 0.05$ **$p < 0.01$ ***$p < 0.001$

IFNγ was the only cytokine that was significantly downregulated in the insulitis lesion as a result of either anti-CD2 or anti-CD8 mAb treatment, indicating a central role for IFNγ as a mediator in the development of diabetes. This is supported by evidence from studies reported in the literature dealing with deletion of this cytokine, either by mAb administration or by the

generation of GKO animals during the development of diabetes (104) or its receptor (IFNγR) (105) (109)(107).

While, I was hoping to identify the cellular source of IFNγ in the development of diabetes by the selective deletion of $CD4^+$ and $CD8^+$ T-cells, the results did not clearly answer this question. Both types of T-cell subsets are known to secrete this cytokine. Because removal of either cell subset resulted in the complete downregulation of IFNγ steady-state mRNA level, this suggests the existence of cross-regulation, or a positive feedback loop between $CD4^+$ and $CD8^+$ T-cell subsets. Thus, the removal of either cell subset can result in breaking this positive feedback loop and the disappearance of IFNγ. However, it remains unknown which cell subset is driving the other. For example, there is evidence supporting the role of $CD4^+$ T-cells as not only helper/inducers of $CD8^+$ T-cells, but also as terminal effectors (see Chapter 1, section V).

IL-2, is more closely related to $CD4^+$ T-cells. This other type I cytokine (known to function also as a proliferative and activating agent for T- and NK cells) has also been reported to have a role in diabetes development (111). In the present study, IL-2 steady-state mRNA level was downregulated in the insulitis lesion after the treatment with anti-CD2 mAb but not after the treatment with anti-CD8 mAb. The disappearance of IL-2 may explain the effectiveness of anti-CD2 mAb treatment on the development of diabetes, as compared with anti-CD8 mAb treatment. It is possible that despite the increased number of $CD8^+$ T-cells that accompanied the anti-CD2

mAb treatment, the $CD8^+$ T-cells may not have been effective in causing disease due to the disappearance of IL-2. On the other hand, IL-2 may have been able to activate the low number of $CD8^+$ T-cells, left after anti-CD8 mAb treatment, or to activate $CD4^+$ T-cells themselves to proliferate and/or to become terminal effectors.

The role of IL-12 is less clear from these studies. While anti-CD2 mAb treatment did not affect the IL-12 steady-state mRNA level, the anti-CD8 mAb treatment and concomitant reduction of $CD8^+$ T-cells, surprisingly, significantly decreased IL-12 steady-state mRNA level. This may only be explained by a positive feedback loop from $CD8^+$ T-cells on the source of IL-12 (i.e. the APC's) to release IL-12. Although it was reported that some macrophage populations also express CD8 molecules on their surface (199), deletion of macrophages by anti-CD8 mAb treatment cannot be considered as a possible explanation for the decreased IL-12 steady-state mRNA level since FACS analysis did not support this possibility. Furthermore, steady-state mRNA levels of other cytokines known to be secreted by APC's (e.g. TNF$\alpha$, IL-1$\beta$, and TGF$\beta$) were not decreased by the anti-CD8 mAb treatment.

In case of the important type 2 cytokine IL-4, anti-CD8 mAb treatment resulted in the significant upregulation of this cytokine in the islets and the spleen, while anti-CD2 mAb treatment led to significant upregulation of this cytokine only in the spleen. Anti-CD8 mAb treatment may have led to the production of IL-4 by $CD4^+$ T-cells, possibly by deleting an inhibitory factor coming from $CD8^+$ T-

cells (e.g. IFNγ). TNFα was again seen to follow the profile of IL-4 (refer to Chapters 3 and 4). The relationship between these two cytokines is not yet understood despite the positive correlation found between them (Fig. 5-10). While there is no evidence in the literature for a common source or function for them, literature concerning autoimmune diabetes suggests a protective role for both IL-4 and TNFα, and this work was recently summarized (188). However, this study was not able to elucidate the nature of the relationship between them.

It still unclear, however, why it was TNFα that was associated with IL-4 and not IL-10, which was downregulated by either anti-CD2 or anti-CD8 mAb treatments. In other words, any treatment that resulted in protection against the development of diabetes led to the downregulation of IL-10, although not as significantly as IFNγ. Although, it is tempting to interpret the findings concerning IL-10 in the context of it being a pro-inflammatory molecule (especially considering the positive correlation between IL-10 and IFNγ, Fig. 5-9), it is also conceivable that IL-10 follows IFNγ expression in an attempt to downregulate the inflammatory cytokine. However, recent literature suggests that IL-10 may play a pro-inflammatory role in the development of diabetes, at least in the insulitis lesion, since transgenic expression of IL-10 by islet β-cells in the NOD mouse accelerated diabetes onset (96) (97).

***In conclusion, the current studies support a major role for IFNγ expression in the islets during the development of***

*diabetes in the DP-BB rat, and a contributing role for IL-2. As IFNγ and IL-2 are type 1 cytokines, it is reasonable to conclude that type 1 cytokine expression in the islets may be necessary for β-cell destruction and diabetes development. The studies also show that type 2 cytokines (IL-4 and IL-10) may <u>not</u> have to be expressed in the islets to prevent β-cell-destructive insulitis. Rather, systemic (splenic) production of IL-4 (not IL-10) and paradoxically TNFα were found to be associated with prevention of diabetes development. Finally, it appears that the need for <u>both</u> CD4+ and CD8+ T-cells to be present in the islets to lead to β-cell destruction may be related to the finding that IFNγ was only expressed in the islets when both CD4+ and CD8+ T-cell subsets were present. This implies that CD4+ and CD8+ T-cell interactions are required for IFNγ production in the islets, and that IFNγ is a common determinant of CD4+ and CD8+ T-cell-mediated cytotoxicity to islet-β-cells.*

# Chapter 6

# Cytotoxic effectors in diabetes: source and function

# I. Introduction:

In chapter 5, the selective deletion of $CD4^+$ and $CD8^+$ T-cells in the DP-BB rat was investigated. Both treatments resulted in the prevention of diabetes. IFNγ steady-state mRNA level emerged as the only cytokine that was significantly downregulated by either treatment. This, in addition to strong evidence documented in the literature, indicated that IFNγ is central in the destruction of β-cells and the development of diabetes (discussed before) . However, it was not determined how this important type 1 cytokine leads to the destruction of β-cells.

## 1. CTL molecules as possible effectors in diabetes :

One of the ways that IFNγ could lead to the destruction of islet β-cells is by activation of cytotoxic lymphocytes (CTL), i.e. $CD8^+$ T-cells. There is strong evidence implicating the $CD8^+$ T-cells as final effectors of islet β-cell destruction. First, the composition of the insulitis in the human diabetic subject was studied (33) and the majority of the T-cells in the infiltrate at the time of diabetes was found to be of the cytotoxic/suppressor phenotype. Second, while adoptive transfer of NOD splenic $CD8^+$ T-cell clones into irradiated young NOD mice did not result in diabetes as did $CD4^+$ T-cell clones (56), the $CD8^+$ T-cells were shown to be cytotoxic to the islet β-cells with the help of $CD4^+$ T-cells. Furthermore, electron microscopic

studies (57) revealed that islet-specific $CD4^+$ T-cells attached closely to islet cells but did not destroy them, in contrast to $CD8^+$ T-cells which showed pseudopodialike protrusions into the islet β-cells but not α- or δ-cells, leading to destruction of only β-cells. Therefore, current evidence suggests that $CD8^+$ T-cells may be involved in both initiation of autoimmune diabetes (58) (59), and as terminal effectors of islet β-cell destruction.

The close contact used by $CD8^+$ T-cells to the target islet β-cells (57) indicates that they employ Fas-FasL and/or perforin-granzyme B mechanisms to destroy the islets. The structure and functions of perforin and granzyme B were reviewed in detail previously in chapter 1. In this chapter, perforin and granzyme B steady-state mRNA levels in the DR and DP-BB rats (treated with control, anti-CD2 and anti-CD8 monoclonal antibodies) will be examined to determine if these molecules are expressed in the islets, and if so, the relationship between their expression and that of type 1 cytokines (e.g. IFNγ) and diabetes development.

**2. Protocol :**

Steady-state mRNA level studies were done on the cDNA samples prepared from islet and splenic mononuclear leukocytes isolated from DR and DP-BB rats (treated with control, anti-CD2, and anti-CD8 mAbs) according to the procedures mention in chapter 5.

**3. Hypothesis :**

**The development of IDDM should be associated with hyperexpression of the cytotoxic molecules, perforin and granzyme, in the islets. The hyperexpression of these cytotoxic molecules should also correlate positively with that the important type 1 cytokine IFNγ.**

# II. Results

## 1. CTL molecule steady-state levels in diabetes:

The steady-state mRNA levels of perforin and granzyme B in the DR, DP-BB rat groups in the islets and spleen is shown in Fig. 6-1. While there was significant upregulation ($p < 0.05$) of perforin message in mononuclear leukocytes from islets of the DP-BB rat (control) group compared with that of the DR-BB group, both mAb treatments which resulted in protection against the development of diabetes, were associated with significant downregulation ($p < 0.01$) of perforin steady-state mRNA level in the islet mononuclear leukocytes. There was no difference between steady-state mRNA level of perforin before and after the onset of diabetes. In addition, no differences were detected in the levels of perforin steady-state mRNA level among the spleens of these rat groups.

Granzyme B steady-state mRNA levels in islet mononuclear leukocytes from the different groups of rats showed a similar profile to that of perforin; however, the differences in granzyme B steady-state mRNA levels in islets from the different groups lacked statistical significance.

**Cytotoxic Molecule mRNA Levels in Islet Mononuclear Leukocytes of Diabetes-Prone (DP) BB rats Treated with Anti-CD2 and Anti-CD8 mAbs**

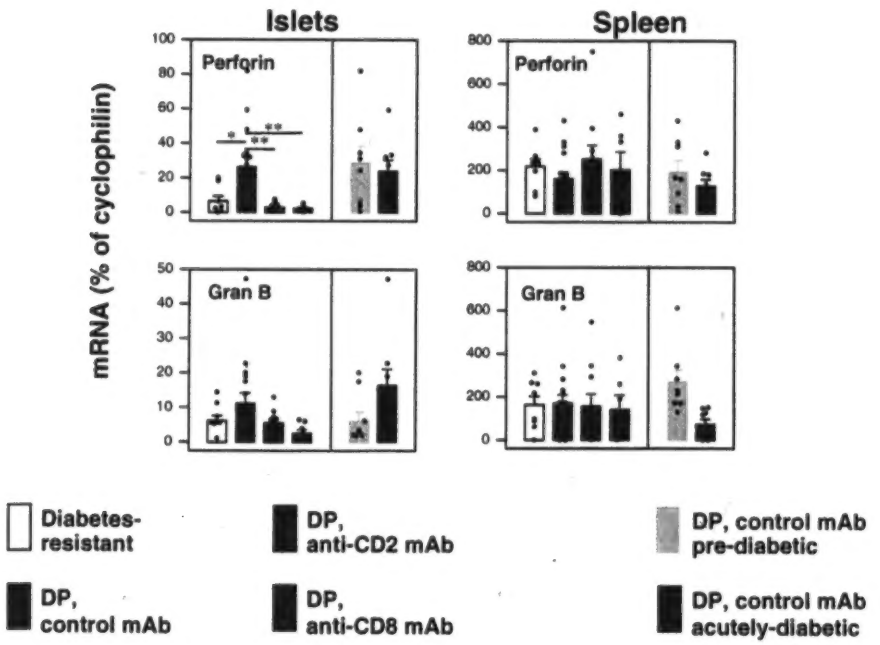



**Fig. 6-1:** Cytotoxic molecule steady-state mRNA levels in mononuclear leukocytes from isolated islets of BB rats, ages 60-75 days. DR-BB rats (n=8, white) are diabetes-resistant BB rats. DP-BB rats were injected daily, starting at the age of 25d, with a control mAb, OX21 (n=16, red), an anti-CD2 mAb, OX34 (n=10, green), and an anti-CD8, OX8 (n=6, blue) monoclonal antibodies. The control group is also broken-down into pre-diabetic rats (yellow, n=8), and acutely-diabetic rats (darker red, n=8) which were studied within 2 days of diabetes onset. Levels of cytokine mRNA (PCR product) are expressed as a percentage of cyclophilin (Cyp). Individual animals in each group are shown as open circles (o). Statistical significance was determined by a non-parametric ANOVA test (Kruskal-Wallis). * $p < 0.05$, ** $p < 0.01$ vs. DP, control mAb-treated group.

**2. Significant correlations between perforin and IFNγ steady-state mRNA levels in islets:**

Fig. 6-2 shows the correlations between expression of the type 1 cytokine IFNγ and the CTL molecules perforin and granzyme B in individual animals. While the steady-state mRNA level of perforin in the islets of BB rats correlated strongly ($r = 0.91$, $p < 0.0001$) with that of IFNγ in the four different groups of animals, the correlation between the steady-state mRNA levels of granzyme B and IFNγ was only moderate ($r = 0.49$, $p < 0.001$). No significant correlation was found between either CTL molecule and IFNγ in the spleen ($r = 0.09$ and 0.30, respectively).

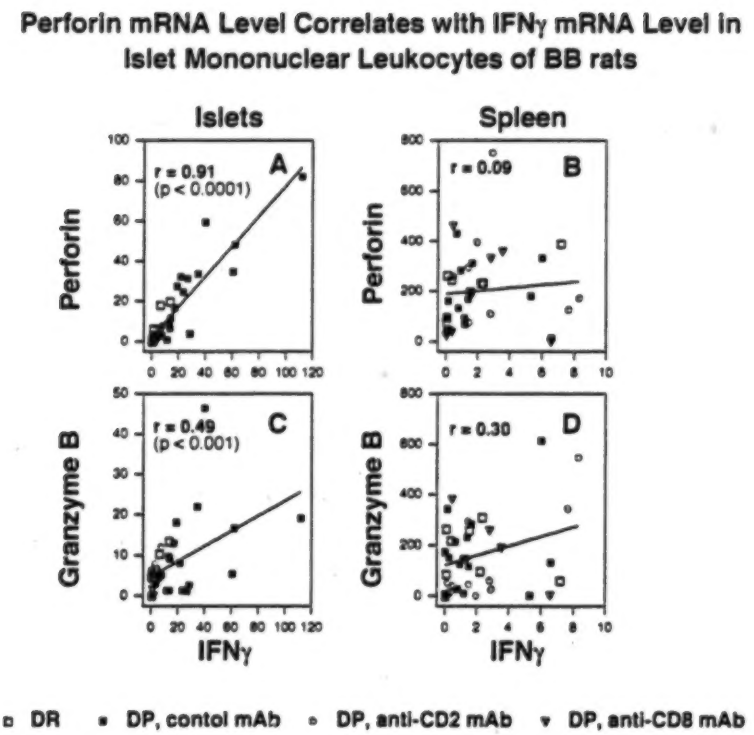



**Fig. 6-2:** Correlations between IFNγ and perforin, and between IFNγ and granzyme B in mononuclear leukocytes isolated from islets and in spleens of individual BB rats: DR (n=8), DP-control mAb, OX21 (n=16), DP-anti-CD2 mAb, OX34 (n=10), and DP-anti-CD8 mAb, OX8 (n=6) treated as described in the legend of Fig. 5-7. The correlation coefficients (r= 0.91($p$ < 0.0001) for A, r= 0.09 for B, r= 0.49($p$ < 0.001) for C, and r= 0.30 for D) were calculated using SigmaPlot v4.11 (Jandel Scientific).

# III. Discussion

The purpose of this study was to 1) investigate the steady-state mRNA levels of CTL molecules (perforin and granzyme B) during the development of autoimmune diabetes in the BB rat, 2) study their relationship to type 1 cytokines (e.g. IFNγ), and 3) to identify the source of these molecules.

The expression profile of these two CTL molecules showed that both perforin and granzyme B steady-state mRNA levels in islet mononuclear leukocytes associated with diabetes development; however, only the changes in perforin steady-state mRNA levels were significant. This implicates perforin in the disease-causing processes. In fact, perforin steady-state mRNA level in the islets was significantly elevated before and just after the onset of disease. Assuming that the steady-state mRNA level leads to similar protein level. This indicates that perforin may play a role in β-cell destruction which is known to precede diabetes onset. At any rate, the significant correlation between IFNγ and perforin steady-state mRNA levels suggests a common regulatory mechanism for both of these genes, and possibly a common functional goal for these two molecules.

The lack of statistical significance in the changes of granzyme B message may be indicative of different storage strategy in the case of

granzyme B, or less of a critical role in β-cell destruction. In other words, the genes of two CTL molecules maybe under different regulatory mechanisms that dictates that perforin gene regulation be more closely associated with that of IFNγ than of granzyme B. In this scenario, IFNγ could be activating perforin expression to affect the target cells in a manner different from the conventional granzyme-B-dependent apoptotic pathway.

The role of perforin in autoimmune diabetes was investigated by crossing perforin-GKO mice with NOD mice (124) and it was determined that perforin-dependent cytotoxicity is not required for the initiation of insulitis but is essential for the destruction of β-cells in NOD mice. A similar model using a granzyme B knock-out, instead of perforin, could test the effect of granzyme B disappearance during the development of diabetes, but this has not been published yet.

Based on the findings of my present studies in the autoimmune-prone BB rat, and the recent literature, a hypothetical scheme (Fig. 6-3) is proposed to explain the relationship of $CD4^+$ and $CD8^+$ T-cells, their cytokine or cytotoxic products, and cross-regulation between these cellular subsets and their effects on the target β-cells.

From the results obtained in the anti-T-cell monoclonal antibody treatment studies, one can see that the protection against the development of diabetes, which was achieved by both mAbs treatments, was associated with downregulation of IFNγ and perforin

steady-state mRNA levels. It was interesting to see that IFNγ steady-state mRNA level was completely downregulated as a result of either $CD4^+$ or $CD8^+$ T-cell removal. This suggests that both $CD4^+$ and $CD8^+$ T-cells must interact in both directions for IFNγ production. In fact, it has been reported that removal of $CD8^+$ cells inhibited IFNγ production by $CD4^+$ T-cells in response to certain stimuli (201) (202).

Furthermore, a more recent study (203) showed that $CD8^+$ T-cells also regulated the generation of perforin-dependent $CD4^+$ cytotoxic T-cells. It was found that perforin-dependent $CD4^+$ cytotoxic T-cells only developed in the absence of activated $CD8^+$ T-cells, which also inhibited the development of IL-4-secreting $CD4^+$ T-cells. While the regulation of the lymphokine profile of $CD4^+$ T-cells by $CD8^+$ T-cells was consistent with mediation by IFNγ (but only in high quantity requiring close proximity), regulation of perforin-dependent cytotoxic activity of $CD4^+$ T-cells seemed inconsistent with mediation by IFNγ and required direct cell contact or close proximity to deliver an unidentified soluble factor. This unidentified factor may be IL-16 (204) reported to be a specific $CD8^+$ T-cell factor known to regulate $CD4^+$ T-cells and to function either through direct contact or as a soluble factor in the form of a tetramer.

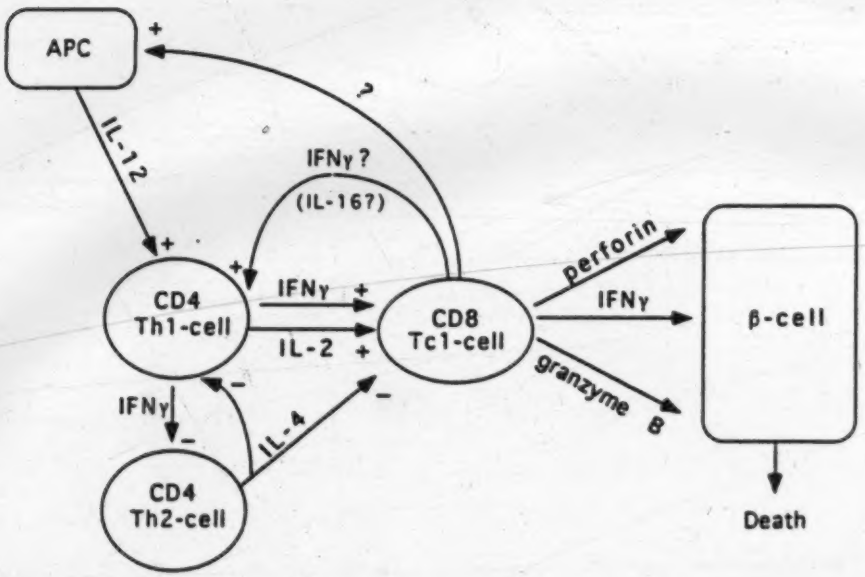
As shown in Fig. 6-3, $CD8^+$ T-cells (Tc1) regulate $CD4^+$ T-cells by the production of IFNγ (201) (202) and this results in the production of IFNγ by $CD4^+$ T-cells in a positive feedback loop. This regulation is consistent with the results obtained in the monoclonal antibody treatment studies in BB rats, since removal of $CD8^+$ T-cells also

results in the disappearance of IFNγ produced by the helper $CD4^+$ T-cells (Fig. 5-7). Also, according to $CD8^+$ T-cell downregulation of the generation of perforin-dependent $CD4^+$ cytotoxic T-cells (203), the presence of perforin in the islets of DP-BB rats may be attributed mainly to $CD8^+$ T-cells. Furthermore, the presence of IL-4 after depletion of $CD8^+$ T-cells in DP-BB rats could be explained by removal of $CD8^+$ T-cells and IFNγ downregulation of $CD4^+$ Th2-type cells (i.e. IL-4-secreting $CD4^+$ T-cells).

Because IL-12 steady-state mRNA level was significantly reduced in islet mononuclear leukocytes from DP-BB rats depleted of $CD8^+$ T-cells (by anti-CD8 mAb), the scheme in Fig. 6-3 shows an unidentified signal that comes from $CD8^+$ T-cells to the APC, which results in the production of IL-12. Upon removal of $CD4^+$ T-cells with anti-CD2 mAb treatment, IL-12 is not affected but IFNγ, IL-2 and IL-4 are removed. Disappearance of IL-2 results in the lack of activation of $CD8^+$ T-cells, i.e. deficient expression of the CTL molecules, perforin and granzyme B. Finally, this scheme does not account for the role of TNFα, which was found to correlate with IL-4 expression and protection against diabetes.

**Fig. 6-3 :**

CD4+ and CD8+ T-cell crossregulation by cytokines:
implications from CD4+ and CD8+ T-cell deletion studies in
autoimmune diabetes-prone BB rats



# Chapter 7

# General discussion and conclusions

## 1. Diabetes then and now:

Understanding the mechanisms responsible for the development of insulin-dependent diabetes mellitus (IDDM, also termed Type I diabetes) remains a challenge. Although several advances have been made in understanding the disease, especially with the employment of recent molecular methodologies, important issues concerning the etiology and exact mechanism of IDDM have not been resolved yet. The disease is thought to result from a complex interaction between the environment of the individual and his/her genetic predisposition. The genetic make-up of the diabetes-susceptible individual is believed to affect the development and/or responsivity of the immune system, possibly to environmental factors, resulting in an autoimmune response that causes the selective destruction of the insulin-producing islet-β-cells.

Autoimmune diabetes is thought to be mediated by different compartments of the immune system. Whereas all leukocytes (T cells, B cells, macrophages, and dendritic cells) may play important roles in the development of autoimmune diabetes, it is the T-cells that have commanded the most attention. Studies using animal models with spontaneous autoimmune diabetes (NOD mice and BB rats) showed that both $CD4^+$ T-cells and $CD8^+$ T-cells are necessary for the disease to occur and be adoptively transferred. The exact role of these T-cell subsets in the development of diabetes is not known; however, our knowledge has been expanded with the help of

the current classification of T-cell subsets based on their characteristic cytokine products.

## 2. IDDM and type 1 / type 2 paradigm :

The current classification of T-cell subsets is based upon their polypeptide products, known as cytokines. In 1986, Mosmann and Coffman (66) reported that the $T_{helper/inducer}$ $CD4^+$ cells, which are considered the regulators of the immune response, can be classified into two distinct subsets: Th1 and Th2. Later, this classification was extended also to $T_{cytotoxic/suppressor}$ $CD8^+$ cells: Tc1 and Tc2 (73). Th1 and Tc1 cells secrete type 1 cytokines (IL-2, IFNγ, and TNFβ) that activate cell-mediated immunity, i.e. cytotoxic and inflammatory responses mediated by T-cells, NK cells, and macrophages. In contrast, Th2 and Tc2 cells secrete type 2 cytokines (IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13) that activate humoral immunity (i.e. antibody production by B cells, particularly IgE responses) and enhance eosinophil proliferation and function. Several other proteins are secreted by both Th1/Tc1 and Th2/Tc2 cells; for example, IL-3, TNFα, GM-CSF, and members of the chemokine families. Also, type 1 and type 2 responses are not the only cytokine patterns possible: T-cells expressing both patterns have been termed Th0 cells, and cells producing high amounts of transforming growth factor β (TGFβ) have been termed Th3. Importantly, the characteristic cytokine products of Th1/Tc1 and Th2/Tc2 are mutually inhibitory for the differentiation and effector functions of the reciprocal phenotype.

Thus, IFNγ selectively inhibits proliferation of Th2 cells, and IL-10 inhibits cytokine synthesis by Th1/Tc1 cells.

***In this thesis, I have studied the steady-state mRNA levels of cytokines during autoimmune diabetes using the BB rat as a model, and I have sought correlations between cytokines, cytotoxic molecules, and pancreatic islet-β-cell destruction.***

The hypothesis I tested stated that the balance between Th1 and Th2 subsets of $CD4^+$ T-cells plays a pivotal role in the development of diabetes: during the progression of IDDM, the Th1 cell production of IFNγ and IL-2 (type 1 cytokines) is upregulated; whereas, Th2 cell production of IL-4 and IL-10, is upregulated with protective treatments that prevent IDDM (Chapter 1, Fig.1-1). This hypothesis also assumed that the mutual inhibitory effect of Th1 and Th2 subsets would reinforce type 1 cytokine domination during IDDM development and type 2 cytokine domination by diabetes preventative therapy.

While testing this hypothesis, I found that it had to be expanded to include other possible sources of cytokines than $CD4^+$(Th1/Th2) T-cells (e.g. macrophages and $CD8^+$ T-cells), and to include other cytokines (e.g. IL-12, considered a type 1 cytokine because it stimulates differentiation of Th1-type cells).

### 3. Evaluation of methodology employed:

In this thesis, I have used a sensitive assay (semi-quantitative PCR) to determine differences in steady-state mRNA levels of these cytokines in the islet inflammatory lesion (insulitis) and in an extra-islet site (the spleen) during the development of spontaneous diabetes and during the administration of protective treatments. Cytokine production is known to occur in minute quantities, and several *in situ* studies reported the level of cytokine production to be close to one positive signal in one thousand cells (205). Indeed, I could not detect cytokine mRNA in islets by Northern blot analysis; however, I found that amplification of cytokine steady-state mRNA level by RT-PCR permitted analysis of cytokine steady-state mRNA levels in islets and spleen.

The benefit of studying cytokine messages is in localizing the activity. Whereas protein products can be transported a long distance to their target location, gene transcription is an activity that occurs within the cellular source and shortly after stimulation. For example, gene expression can be induced within seconds of changing the cell shape by physical forces (206). There are several examples of genes that are known to respond by transcription immediately after stimulation. Transcription factors (e.g. *c-jun* and *c-fos*) (207), and cytokine genes are such examples (208). Therefore, when the message, or steady-state mRNA level, of a cytokine gene is upregulated in a particular immunological site, it can be concluded that this upregulation of message is a function of stimulation confined to the

location. Indeed, the results of this thesis show that the cytokine message profile may be different for the same cytokine at the same time in two different locations such as the islets and the spleen.

Confirmation of translation of transcribed mRNA to protein product, and binding to the target cell are all still necessary to ensure that the message detected corresponds to the final bioactivity of the protein. In fact, these experiments are currently performed in our laboratory in order to correlate cytokine protein expression with that of cytokine messages.

**4. IDDM and type 1 cytokines :**

From the studies reported in this thesis, one can see some important trends. First, when comparing the levels of cytokine messages found in the islets of the DR-BB versus those of the DP-BB rats, there appears to be a general trend of an increase of type 1 cytokines in the latter. This is very evident in the particular case of the prototype type 1 cytokine, IFNγ. Furthermore, this increase was not simply due to an infiltration of leukocytes into the islets. Thus, in all treatments that resulted in protection against IDDM (e.g. CFA), this protection was associated with downregulation of IFNγ message despite the fact that the number of leukocytes in the islets did not significantly differ before the onset of disease. This was evident from histological examination and isolation of similar number of leukocytes from diabetes-protected and control animals. In the case

of other type 1 cytokines, IL-2 and IL-12, correlation with disease was not as strong or lacked statistical significance. Although this indicates that type 1 cytokines as a group, may not be critical in disease development, it reinforces the significance of IFNγ as an important cytokine in diabetes development.

Certainly the role of IFNγ in the development of diabetes is supported by several studies the literature. This evidence can be summarized as follows: 1) Addition of IFNγ, alone or in combination with other cytokines like TNFα/β or IL-1, to pancreatic islets *in vitro* (in picomolar concentrations) inhibited insulin secretion and resulted in destruction of the islets, respectively (78) (209), 2) Local expression of IFNγ by the islet-β-cells (i.e. transgenic mice) has showed that pancreatic expression of this important type 1 cytokine is capable of inducing insulitis and diabetes in nondiabetes-prone mice (89), 3) Deletion of IFNγ by IFNγ gene-targeted-disruption (GKO) in the NOD mouse showed that the absence of IFNγ delays the destruction process of the β-cells and the development of diabetes (104), and mutation of the gene encoding the IFNγ receptor was reported to markedly inhibit insulitis and completely prevent diabetes development (105). Also, deletion of IFNγ by administration of anti-IFNγ monoclonal antibody in both NOD mice and BB rats (107) (108) (109), or by administration of soluble IFNγ receptor (110), led to protection against diabetes.

In support of the IFNγ results reported here, Kolb and colleagues also reported that the level IFNγ message increases in the

pancreases of BB rats as they grow from 50 to 70 days of age in comparison with the non-diabetes-prone Wistar rats (210). In addition, another study (211) reported that in DP-BB and RT6-depleted DR-BB rats the levels of IFNγ and IL-12p40 mRNA increased with disease progression. Incidentally, both of those studies utilized RT-PCR as I did in my studies.

It is important to note that the association of IFNγ with disease progression is not necessarily a systemic phenomenon but localized to the insulitis lesion. This was evident when the profile of cytokine steady-state mRNA levels in the islets was compared to that in the spleen. Thus, IFNγ steady-state mRNA level was consistently downregulated in the islets of DP-BB rats protected from diabetes (by CFA, and anti-T-cell mAb treatments), whereas only CFA treatment decreased IFNγ mRNA levels in spleen.

**5. IDDM and type 2 cytokines :**

In case of type 2 cytokines in the development of diabetes, it was more difficult to reconcile the results obtained here with our initial hypothesis (Chapter 1, section VI). Whereas in the initial hypothesis protection from diabetes was postulated to occur through the upregulation of type 2 cytokine which are known to downregulate type 1 (especially IFNγ), the results of my studies show that this was not necessarily the case. Generally, protection was achieved in my studies independent of type 2 cytokine upregulation.

However, it is important to point out that the expression of the two type 2 cytokines investigated, IL-4 and IL-10, was different in the studies reported here. Therefore, I will discuss each cytokine independently of the other.

The role of IL-4 in the protection against the development of diabetes is well documented in the literature. For example, transgenic expression of IL-4 by islet-β-cells of the diabetes-prone females NOD mice was effective in the prevention of insulitis and diabetes (93). Furthermore, the expression of IL-4 in the pancreas alone was effective in the induction of functional tolerance to islet antigens reflected by the inability to reject syngeneic pancreatic islets transplanted into IL-4-transgenic NOD mice and the failure of diabetogenic spleen cells to induce diabetes in NOD-IL-4 recipients. Also, systemic administration of either IL-4 (98), or anti-CD28 monoclonal antibody (212), which preferentially stimulated the intra-islet production of IL-4, protected against diabetes development in NOD mice.

The expression of IL-4 in the islets is thought (213) to provide protection against destruction of the islet β-cells through influencing the reactivity of diabetogenic T-cells encountered within, thereby rendering them unable to mediate disease. Alternatively, IL-4 could be acting through tissue localized APCs. IL-4 was shown (214) to reduce the expression of FcγRI on macrophages limiting their ability to take up and present tissue antigen to T-cells. It is important to

note that the studies relating to IL-4 were conducted on NOD mice, which may have immune mechanisms different from those responsible for spontaneous IDDM in the BB rat.

Contrary to the literature on the NOD mouse and the initial hypothesis, the results of this thesis show that IL-4 steady-state mRNA level was significantly upregulated in the insulitis lesion during diabetes prevention provided by only one treatment, anti-CD8 mAb treatment. In other words, protection from diabetes was achieved by two other treatments (CFA and anti-CD2 mAb administration) without significant upregulation of IL-4 in the insulitis lesion. It is conceivable that the protection of IL-4 may not be exerted at the target cell level, rather IL-4 may correct the bias toward type 1 cytokines systemically. Indeed, IL-4 was found to be significantly upregulated in the spleens of the DP-BB rats after both anti-CD8 and anti-CD2 mAb treatments which protected against the development of diabetes. IL-4 could be providing protection against an autoimmune response by interfering with antigen presentation by the APCs to the T-cell populations in the spleen (as mentioned above), rather than downregulating type 1 cytokines in the islets.

The role of IL-10 in autoimmune diabetes, on the other hand, appears paradoxical. Some literature supports a protective role for IL-10. For example, the injection of human recombinant IL-10 (99), or the non-cytolytic IL-10/Fc fusion protein (100) into NOD mice

provided protection against the development of diabetes in this diabetes-prone animal model. On the other hand, transgenic expression of IL-10 by the pancreatic islet-β-cells led to massive inflammation of the pancreas in diabetes-resistant mice (95) and acceleration of β-cell destruction in diabetes-prone NOD mice (96), requiring only the presence of NOD MHC genes but not necessarily the *idd* genes (97). Therefore, it appears that the protection from diabetes is associated with the presence of IL-10 systemically, whereas local production of IL-10 in the pancreas and the islets leads to inflammation and destruction of the islets β-cells.

The profile of IL-10 steady-state mRNA levels studied in this thesis also reveals this paradoxical effect of the cytokine. On the one hand, IL-10 steady-state mRNA levels in the insulitis lesion in all the studies reported here correlated positively with that of IFNγ, suggesting a pro-inflammatory role for IL-10. IL-10 has multiple cell sources, including T-cells, NK cells (215), and macrophages (216). Macrophages and NK cells are known to move into the insulitis at the later stages of the disease in the BB rat (162). This may explain why the presence of IL-10 associates with destructive processes of the β-cells. In addition, IL-10 may be mediating its effects indirectly by altering antigen processing and presentation in the pancreatic environment through enhancing the expression of MHC class II antigens on resting B cells (217).

On the other hand, the close relationship between IL-10 and IFNγ may not be due to a common functional goal (i.e. inflammation)

but may simply reflect the attempt of IL-10 to downregulate IFNγ. It was reported that IL-10 monoclonal antibody, when administered to NOD mice, did not reduce the incidence of diabetes (99), indicating that IL-10 did not promote the development of diabetes.

In my studies, we also saw the upregulation of IL-10 steady-state mRNA level in the spleens of the diabetes-resistant (DR) BB rats compared with that of diabetes-prone (DP) BB rats. Furthermore, CFA protection of the DP-BB rat against IDDM was associated with a significant upregulation of IL-10 steady-state mRNA level in the spleen. This indicates that the presence of IL-10 systemically is associated with protection possibly by inhibiting macrophage costimulatory activity by selectively inhibiting the upregulation of B7 expression (218).

In conclusion, although more experiments are needed to elucidate the mechanisms by which type 2 cytokines may prevent spontaneous autoimmune diabetes in the BB rat, both IL-4 and IL-10 may exert their effects systemically rather than locally in the islets.

**6. TNFα and diabetes :**

TNFα is known to be cytocidal for tumor cells. It was found to be cytostatic (alone) and cytotoxic (in combination with IFNγ and IL-1) to β-cells *in vitro* (78) (209). But contrary to the *in vitro* findings, TNFα is associated with protection *in vivo* during the development

of diabetes. Expression of TNFα and TNFβ transgenes in the islets showed that these cytokines lead to insulitis but not diabetes in non-diabetes-prone mice (185). Furthermore, transgenic expression of TNFα by islet-β-cells prevented the development of autoimmune diabetes in NOD mice (92). In that study, the local production of TNFα in the islets did not merely prevent the activation of islet specific T-cells but also led to antigen-specific $CD4^+$ T-cells tolerance and increased local expression of Th2 cytokines (e.g. IL-4). Furthermore, the administration of TNFα to NOD mice transplanted with syngeneic islets (219) prolonged islet graft survival and downregulated type 1 cytokines in the islet graft and the spleen. Also, expression of IL-10 in TNFα-treated mice was not upregulated in the islets but was elevated in the spleen.

TNFα may decrease the generation of autoreactive T-cell effector function and inhibit the development of diabetes by reducing signaling through the TCR (220). Both IL-4 and TNFα were associated with protection against the development of diabetes and these two cytokines were positively correlated in individual animals in my studies. The mechanisms by which IL-4 and TNFα protect against diabetes may differ. While IL-4 can confer protection by immunoregulation during both the initiation and the ongoing phases of the disease process, TNFα completely blocks the disease initiation but is unable to prevent diabetes-prone mice from succumbing to diabetes development when diabetogenic spleen cells from NOD mice are introduced (188).

## 7. Type 1 cytokines and CTL molecules:

As discussed above (section 4: IDDM and type 1 cytokines), the correlation between the type 1 cytokine, IFNγ, and diabetes development is quite consistent. Also I found that the profile of the steady-state mRNA levels of the CTL molecules, perforin and granzyme B resembled that of IFNγ. Furthermore, there was a highly positive correlation between IFNγ and perforin messages in the insulitis lesion. This suggested that perforin could be an important terminal effector molecule in the destruction of the β-cells. This is supported by GKO studies investigating the role of perforin in β-cell destruction and diabetes in the NOD mice (124).

The relationship between the steady-state mRNA levels of type 1 cytokines and CTL molecules granzyme B and perforin was demonstrated by immunopharmacological studies using pentoxifylline (PTX), a PDE inhibitor which was found to selectively downregulate the biosynthesis of Th1 cytokines while leaving Th2 cytokine production intact (196). This drug was also found to inhibit expression of the effector CTL molecules granzyme B and perforin (134), by anti-CD3-antibody activated T-cells *in vitro* . This, in addition to the knowledge that IL-2 and IFNγ are known to enhance cytotoxic functions of different immune cells (e.g. $CD8^+$ T-cells and NK cells), leads to the conclusion that expression of CTL molecules is directly related to type 1 cytokine production.

Whereas the anti-T-cell mAb studies revealed a strong correlation between the steady-state mRNA levels of perforin and IFNγ ( Chapter 6), the correlation between granzyme B and IFNγ was not as strong. Recent literature indicates that while both perforin and granzyme B are essential for the mechanism of apoptotic death, perforin appears necessary for cell lysis (129). It remains to be established whether pancreatic islet-β-cells die by apoptosis and how important granzyme B for this process during spontaneous autoimmune diabetes.

Other possible candidates may be involved in β-cell destruction such as Fas-FasL interactions and free-radical production; both functions are known to be enhanced by IFNγ production. Therefore, our lab is currently investigating the possibility that Fas-FasL is involved in the destruction of β-cells, as well as the role of free-radical production by macrophages.

**8. Interaction between T-cell subsets and their effects on the β-cells :**

The major conclusions from this thesis so far are : 1) The type 1 cytokine IFNγ is a key cytokine in the development of autoimmune diabetes, and any treatment or intervention that leads to protection against the development of disease is associated with downregulation of IFNγ, 2) The CTL molecule, perforin, strongly associates with IFNγ,

hence its candidacy as a terminal effector molecule involved in β-cell destruction.

Fig. 6-3 offers a possible explanation for the relationship between the cytokines, their possible source, and cross-regulation. The scheme in this Figure shows that IFNγ, from $CD4^+$ or $CD8^+$ T-cells, is the central mediator for the development of diabetes. When IFNγ is absent or downregulated, the β-cells are spared from the destruction by the effector molecules (perforin, granzyme B, and IFNγ); in addition, IL-4 may then be expressed by Th2 cells. However, the protection against β-cell destruction is primarily due to the absence of IFNγ not the presence of IL-4. Whereas this scheme can explain the role of IFNγ and IL-4 during the development of diabetes, it does not account for IL-10 or TNFα. The fact that IL-10 could be produced by other sources (e.g. macrophages or NK cells) or under different regulatory mechanisms from those governing IL-4 needs further investigation to be able to place it in this scheme. Similarly, the source and regulation of TNFα needs to be identified and its relationship to the production of IL-4 needs to be defined. It is also important to remember that there may be other sources of these cytokines (e.g. the IL-4-secreting NK1.1-Tcells (221)).

**9. The reliability and reproducibility of results in this thesis :**

The results presented throughout the thesis were based on single studies. Therefore, the reproducibility of the results may be a matter of concern. However, due to the homogeneity of the diabetes-prone animals and reproducibility of the protocols used in these studies, it can be reasoned that the results obtained here are reliable. The animals used in the current studies are BB/Wor rats, which were obtained directly from a highly inbred colony. The animals were raised under strict pathogen-free conditions. The same animal caregivers were involved and their care was consistent throughout the studies. Treatments were administered regularly according to strict schedules. Results obtained with respect to diabetes incidence and monoclonal antibody depletion of leukocyte subsets in our studies were similar to those obtained by the previous investigators. The results of gene transcription studies obtained here were also consistent. By examining the expression profile of IFNγ alone, for example in the DR- and DP-BB rats (Appendix 5), one can see that the pattern of expression is consistent. In addition, the minimum number of animals in each group studied was six. Therefore, it is likely that the results can be reproduced if repeated using the animals from the same inbred BB/Wor colony, raised under the same conditions, and following the same protocols.

## 10. Future directions :

The first important question is whether the messages of cytokine genes (steady-state mRNA levels) measured in these studies are translated into corresponding cytokine protein production. Therefore, it will be necessary to identify the cytokine proteins that are expressed in the islets of BB rats and to associate these with diabetes development. The second important question is the source of cytokine message. Therefore, immuno-histochemical studies co-localizing the cell source and product will be required to identify the source of cytokines and/or cytotoxic molecules. The studies proposed can employ the use of antibodies recognizing a unique cell-surface marker and the cytokine or cytotoxic molecule in question. We have successfully carried out this procedure in our lab on the NOD mouse system (222), and we are currently in the process of using the same technology for the BB rat studies.

The CFA study showed that it was possible to prevent the development of diabetes at least partially, and that this prevention was associated with the downregulation of type 1 cytokines, particularly IFNγ. However, this downregulation did not appear to be the result of type 2 cytokine upregulation. Therefore, the question remains as to how CFA downregulated IFNγ. Although, the type 2 cytokines, IL-4 and IL-10, may play a role (223) in the NOD mouse model, it seems that TNFα may be the mediator of the protective effect of CFA in the BB rat (187). Studies identifying the role and source TNFα in CFA-mediated protection against autoimmune

diabetes, as well as the mechanism by TNFα which blocks initiation of disease, are needed.

In addition to the conventional $CD4^+$ or $CD8^+$ αβT-cells, other cellular subsets may also play a role in the development of diabetes. For example, some studies (162) reported the presence of $CD4^-/CD8^-$ T-cells in the insulitis of the BB rats. These double negative cells ranked second to $CD4^+$ T-cells in abundance in the insulitis lesion of the DP-BB rat. It maybe of interest to identify the cytokine profile of these cells and define their role during autoimmune diabetes. It was postulated that these double negative T-cells provide help to B-cells in generating pathogenic anti-glomerular basement membrane antibodies. Also, the role of B-cells in the development of diabetes is now being investigated and it is becoming clear that B-cells may also play an important role in disease initiation (224).

It will also be interesting to investigate whether a diabetes-protective role may be played by cells other than the conventional Th2 lymphocytes. For example, a small number of splenic CD8 γδT-cells were found (225) to play an important regulatory role in protecting NOD mice from diabetes following aerosol insulin administration. Spleens from these mice were found to be rich in IL-4, IL-10, and TGFβ.

Other cytokines that may relate to Th1/Th2 balance can also be studied in the BB rat. For example, IL-18 (IFNγ-inducing factor, IGIF) has been reported to associate with disease development in NOD mice

(226). In addition, the role of IL-6 in directing the differentiation of IL-4-producing $CD4^+$ T-cells was reported (227). Studying the profile of IL-6 message in the DP- and DR-BB rats and correlating that with IL-4 message under protective condition may also prove helpful in elucidating the mechanisms underlying protection.

Finally, most therapies to prevent autoimmune destruction of islet β-cells have centered around giving the type 2 cytokines, IL-4 and IL-10, alone or in combination. The treatment of other autoimmune disease (e.g. inflammatory bowel disease, IBD) with IL-10 alone showed some success (228). Similar results were obtained with IL-4 and it was expected to correct the immune deviation in inflammatory disease (229). Based on the results from this thesis, in addition to recent reports in the literature, the combination of IL-4 and TNFα should yield better results than IL-4 and IL-10, separately or in combination, in protecting individuals against diabetes.

***In conclusion, the studies reported in this thesis show that the development of autoimmune diabetes in the DP-BB rat is associated with immune deviation reflected by upregulation of type 1 cytokines (especially IFNγ) in the insulitis lesion. It also provides candidates (e.g. perforin) for the mechanism by which this immune deviation results in islet-β-cell destruction. The protection of islet-β-cell against destruction conferred by therapeutic interventions is the result of downregulation of type 1 cytokines (especially IFNγ), but not necessarily through type 2***

*cytokine upregulation. And finally, several questions and future possible experiments are posed based on the findings reported here.*

# Chapter 8

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# Appendix 1

## Preparation of Con-A-activated DR-BB Splenocytes

For the preparation of the positive control cDNA sample, the spleens of 2 male and 2 female DR-BB rats were collected at the age 0f 110 days and meshed into single-cell suspension of RPMI (10% FCS) or complete medium using a tissue metallic screen. The cell suspension was washed twice with 15 ml RPMI (10% FCS) and the total number of viable cells was counted (see below). Total cell preparations were plated at a rate of 0.5 x $10^6$ cells/ml to a total of 10 mls of RPMI (10% FCS) in each plate. Concavalin-A from ICN (Con-A HC) was added at a concentration of 5 µg/ml (i.e. 50 µg/plate); 10 plate were used for each spleen. The plates were cultured at 37°C (5% $CO_2$) in a humidity chamber for 48 hours. The cultures were pooled into 15-ml conical tubes and spun down at 1800 rpm in a clinical centrifuge for 5 minutes at room temperature. The supernatant was kept for cytokine analysis. The pellets (i.e. cells) were washed twice with 15 ml RPMI(10% FCS). The cells were resuspended in RPMI (10% FCS) and counted with the help of a haemocytometer chamber. The cell suspension was run on a Ficoll gradient ("HISTOPAQUE-1077" for MNC isolation from Sigma Diagnostics) by mounting 9 mls of cell suspension, containing 2 x $10^6$ cells, on 3 mls of Ficoll in 15-ml conical tubes. the tubes were spun at 1200 rpm for 20 minutes with no brake in a clinical centrifuge (ICN). The interface ring containing the MNC's (leukocyte suspension) was harvested and washed twice with RPMI then the cells were counted and stained for viability as before.

# Appendix 2

## Calculations for PCR amplifications:

Number of copies (C) at the end of 40 cycles (n) with 100% efficiency of PCR = $2^{n-2} = 2^{38}$ (assume that the dsDNA target sequence forms in the third cycle)

Therefore, C at 80% efficiency of PCR = $1.8^{38}$ copies

= $5 \times 10^9$ copies

Molecular weight of product ($\mu$) =

number of copies (C) x number of base pairs (bp) x ((660 g/mol)/1 bp)

= $5 \times 10^9$ copies x 300 bp x (660 g/mol)/1 bp = $9.9 \times 10^{14}$ g/mol

Mass of PCR product (m) = n x $\mu$ = (1 mol/6.02 x $10^{23}$ molecules) x $9.9 \times 10^{14}$ g/mol = $1.6 \times 10^{-9}$g = 1.6 ng.

# Appendix 3

## Preparation of hybridoma supernatants for injection:

### 1. Screening Assays:

The 1 ml aliquot of the hybridoma supernatant from all the different batches were thawed and the following tests were performed:

1. For Mycoplasma-free supernatant by a biological test kit (Sigma).
2. For Endotoxin-free supernatant by Endotoxin kit from (Sigma).
3. For total protein by a MicroBCA protein assay kit (Pharmacia).
4. Indirect immunofluorescence by FACS to determine the percentage of positive cells which stained with each specific monoclonal antibody (OX8, OX21, and OX34) from Ficoll splenocyte-cell preparation of Wistar-Furth (WF) rat.
5. ELISA to determine the total amounts of IgG1 in OX8 and OX21 hybridoma supernatants and IgG2a (OX34) hybridoma supernatant.

**Results :**

The selected supernatants used for injection were :

1. Mycoplasma-negative.
2. The level of endotoxin was 0.01-0.05 ng/ml (below the normal of 1 ng/ml).
3. The amounts of total protein were 1,040 µg/ml, 1,180 µg/ml and 1,590 µg/ml for OX8, OX21 and OX34 TCS, respectively.
4. The staining on WF splenocytes was successful and highly specific (80 ± 4% for OX34, 36 ± 6% for OX8, 2 ± 1% for OX21, and 2 ± 1% for control Ab IgG1 and IgG2a )

5. The amounts of IgG1 in OX8 and OX21 hybridoma supernatants were 59.8 µg/ml and 80 µg/ml, receptively. The amount of IgG2a in OX34 hybridoma supernatant was 106.5 µg/ml.

Therefore, taking into account that each rat was injected i.p. with 2 mls of supernatant hybridoma daily, the amount of IgG1 injected daily in case of :

| | | | | |
|---|---|---|---|---|
| OX8 | (isotype IgG1) treatment | = 2 ml x 59.8 µg/ml | = | 119.6 µg |
| OX21 | (isotype IgG1) treatment | = 2 ml x 80 µg/ml | = | 160 µg |
| OX34 | (isotype IgG2a) treatment | = 2 ml x 106.5 µg/ml | = | 213 µg |

These amounts represent the following percentages of the total protein injected, in case of :

| | | | | |
|---|---|---|---|---|
| % OX8 | = | (59.8 µg/1,040 µg) | x 100 = | 5.75 % |
| % OX21 | = | (80 µg/1,180 µg) | x 100 = | 6.78 % |
| % OX34 | = | (106.5 µg/1,593 µg) | x 100 = | 6.69 % |

# Appendix 4

## ELISA protocol:

Three monoclonal antibodies were used for injecting the BB rats :

- OX8, IgG1 mouse anti-rat
- OX21, IgG1 mouse anti-human
- OX34, IgG2a mouse anti-rat

The steps for the ELISA to determine the concentration of the IgG's in hybridoma supernatants of the aforementioned monoclonal antibodies were as follows:

**a. Coating :**

The primary antibodies (goat-anti-mouse IgG1, goat-anti-human IgG1, and goat-anti-mouse IgG2a purchased from Sigma for OX8, OX21, and OX34, respectively) were prepared for coating by diluting the stock in 1% BSA/PBS to give 1.0 µg/ml. The solution was mixed well in a sterile polypropylene tube by inverting gently several times. 100 µl of the diluent were aliquoted in each well, in a 96-well ELISA plate, (avoiding the outside wells) and incubated overnight at 4°C for best results.

The coating was disposed of by flicking the plate above the sink and washing with 0.05% Tween-PBS (PBST) while rotating plate for 3-5 seconds using the ELCAWASH setup. The plate was rotated 180° and washed again. The wash was repeated twice and the plate was dried by flicking plate onto paper towel.

**b. Addition of samples/standards :**

25 µl of samples (OX-8, OX21, and OX34 supernatants) were serially (1:1) diluted 16 times in RPMI phenol-red-free medium. For negative control, 25 µl of tissue culture medium (RPMI + 1% FBS) alone was used instead of the sample.

The standards used were a mouse IgG1 (for OX8 and OX21) and IgG2a (for OX34) from Sigma, which were diluted in similar fashion starting at 1000 µg/ml. Serial dilution were carried down to 1.53 x $10^{-2}$ µg and two standard curves per plate were run each time (in dilution in duplicate). The level of detection was about 8 µg/ml.

**c. Blocking :**

This step was important to block the endogeneous HRP activity that may be in the hybridoma supernatants. The $H_2O_2$ stock (30%) was diluted to 1% by adding 100 µl to 2900 µl PBST and 25 µl were added to each well. The reaction was left at RT for 10 minutes then the wells were washed once with PBST.

**d. Addition of secondary antibody :**

50 µl of biotinylated-HRP goat anti-mouse IgG from Sigma (1:1000 of 1%BSA, PBST) were added to each well (in case of OX21, a mouse anti-human biotinylated-HRP IgG). The plates were incubated at 14°C for 45 minutes

**5. Substrate addition:**

The following solution was made up: 9.4 ml $dH_2O$, 100 µl (tetramethylbenzidine blue) TMB/DMSO mix, 500 µl Na Ac, and 1 µl 30% $H_2O_2$. 80 µl were added to each well, and plates were incubated

for 15 minutes at room temperatre with shaking (Blue color appeared soon).

**6. Stopping solution :**

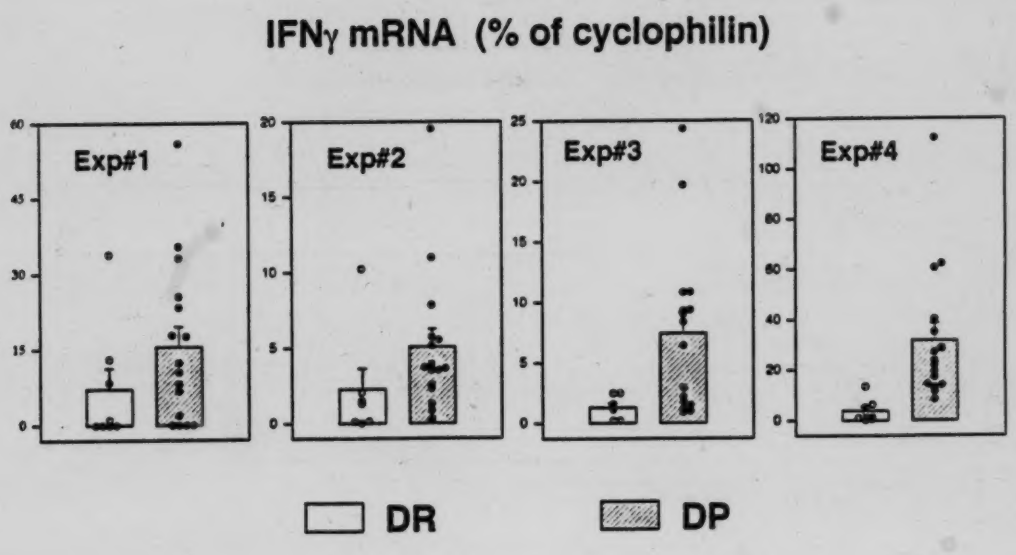
40 µl of 1:20 dilution of 12M $H_2SO_4$ were added and color turned yellow.

**7. Reading:**

The plate was inserted into the ELISA plate reader (Molecular Devices, $V_{max}$ at 450-570nm) and the results were quantitated using the Softmax software to calculate the concentraions of antibody (i.e. O.D. vs. antibody).

# Appendix 5

The following is the gene expression profiles of the cytokine IFNγ in the DR- and DP-BB rats in the four studies used in this thesis. The expression profiles appear similar in the four studies although each study contains a different lot of rats. Keeping in mind that these rats are obtained from a highly inbred colony and kept under the same strict conditions, this indicates consistency even at the level of gene expression from one experiment to the next.




Exp#1: CFA study
Exp#2: Theo Study
Exp#3: anti-CD8 study
Exp#4: anti-CD2 study